AD_____

GRANT NUMBER DAMD17-96-1-6076

TITLE: Stimulation of p53-dependent Transcription by the Growth Suppressor, c-Abl

PRINCIPAL INVESTIGATION: Xuan Liu, Ph.D.
John Cogan

CONTRACTING ORGANIZATION: University of California, Riverside
Riverside, California 92521

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 2

19991208 192

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jun 98 - 31 May 99)

4. TITLE AND SUBTITLE
Stimulation of p53-dependent Transcription by the Growth Suppressor, c-Abl

5. FUNDING NUMBERS
DAMD17-96-1-6076

6. AUTHOR(S)
Xuan Liu, Ph.D.
John Cogan

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Riverside
Riverside, California 92521

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Growth suppressor c-Abl interacts with p53 in response to DNA damage and over-expression of c-Abl leads to G1 growth arrest in a p53-dependent manner. Here, we show that c-Abl binds directly to the carboxyl terminal regulatory domain of p53 and that this interaction requires tetramerization of p53. Importantly, we demonstrate that c-Abl stimulates the DNA-binding activity of wild-type p53 but not of a carboxyl-terminally truncated p53 (p53Δ363C), and furthermore, a deletion mutant of c-Abl that does not bind to p53 is also incapable of activating p53 DNA-binding, suggesting that the binding to the p53 carboxyl terminus is necessary for c-Abl stimulation. To investigate the mechanism for this activation, we have also shown that c-Abl stabilizes the p53-DNA complex. Interestingly, the stimulation of p53 DNA-binding by c-Abl does not require its tyrosine kinase activity, indicating a new function for c-Abl. Together, these observations provide evidence for a role of c-Abl as a growth suppressor protein in activation of p53 DNA-binding via the carboxyl terminal regulatory domain and tetramerization.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	52
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

6/20/93

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

Loss of cell growth regulation is a characteristic of cancer cells. To achieve our goal of designing therapies for cancer, we must understand how cancer proteins affect cell growth. The aim of this proposal is to address this question for the cancer related proteins, p53 and c-Abl. Specifically, we have proposed:

3. To define the domains on p53 required for c-Abl binding
2. To examine the effect of c-Abl on the DNA-binding activity of p53
3. To characterize the effect of c-Abl on p53-dependent transcription *in vitro*
4. To determine the effect of Gal4-Abl on transcription from a promoter containing Gal4 sites
5. To examine whether general transcription factors are phosphorylated by c-Abl

BACHGROUND AND PREVIOUS WORK

p53 is an important tumor suppressor gene, mutated, or absent, in over 50% of all cancers studied. It functions as a sequence-specific DNA-binding transcription factor. In response to double-stranded DNA breaks p53 is converted from a latent to an active form. This results in increased expression of p53-responsive proteins such as p21 which are required for growth arrest at the G1 to S phase transition. It also mediates apoptosis via the increased expression of proteins such as Bax. Inactivation of p53, therefore, results in the loss of a cell cycle checkpoint required for repair of damaged DNA and prevents apoptosis in response to severe DNA damage. In the absence of these responses oncogenic mutations can accumulate which may result in tumor progression.

Based on the G1 arrest phenotype of p53, we reasoned that p53 transcriptional activity must be affected by the cell cycle proteins which regulate G1. c-Abl has been reported to be a growth suppressor and overexpression of c-Abl leads to G1 growth arrest in fibroblasts. The c-Abl protein is a predominantly nuclear tyrosine kinase. The kinase activity of c-Abl is tightly regulated *in vivo*, possibly by binding to unidentified inhibitory proteins, and is required for c-Abl to suppress growth. Links between the c-Abl proto-oncogene and cell cycle suggest that c-Abl normally acts as a negative regulator of cell growth and that it may function through p53. The availability of mouse fibroblasts containing disruptions of the Rb or p53 genes allowed us to genetically test this possibility. Our results show that c-Abl requires p53 but not Rb to suppress growth. In addition, we also find that c-Abl binds to p53 *in vitro* and enhances the ability of p53 to activate transcription from a promoter containing a p53 DNA binding site in a transient transfection assay. Deletion of the p53 binding domain in c-Abl (ΔProl, a deletion of proline rich domain, aa 793-1044) impairs the ability of c-Abl to stimulate p53 transcriptional activity and to suppress growth. These results

suggest that the regulation of p53 transcription is very important in negative growth control by c-Abl. Therefore, a detailed understanding of how c-Abl stimulates p53-dependent transcription may allow the rational design of therapies which can reactivate the Abl-p53 pathway in tumor cells, resulting in cell cycle arrest and apoptosis of tumor cells.

Towards this end, during the first year of the grant (7/96 to 6/97), we constructed a set of deletions to disrupt the domains of p53 responsible for nuclear localization (aa 316 to 322), tetramerization (aa 325 to 356) and the regulation of the DNA binding activity of p53 (aa 363 to 393). Furthermore, the ability of these mutants to interact with c-Abl was also tested using a GST pull-down assay. Our results show that deletion of last 30 amino acids in p53 severely disrupted its ability to bind to c-Abl and deletion of the tetramerization domain also greatly reduced the binding to c-Abl. Based on these results, we propose a model in which c-Abl interacts with the regulatory domain (aa 363 to 393) in p53 to diminish its negative regulatory effect and to enhance the DNA binding activity of p53. This interaction, however, requires the tetrameric conformation of the protein. To test this requirement, ability of a mutant p53 (341K344E348E355K, tetramerization impaired) to interact with c-Abl was investigated. Our results show this mutant is defective in c-Abl interaction. These data led us to focus our studies on c-Abl's ability to enhance the DNA binding activity of p53.

During the second year of the grant (7/98 to 6/99), we expressed c-Abl with baculovirus system and showed that partially purified c-Abl stimulates p53 DNA-binding activity. In order to test our model that c-Abl interacts with the negative regulatory domain to enhance the DNA binding activity of p53, highly purified c-Abl was required. To overcome this problem, a GST-Abl baculovirus was employed and a large amount of purified protein was obtained.

RESULTS

In current budget year (7/98 to 6/99), we have obtained following results:

c-Abl stimulates p53's DNA-binding ability by decreasing off-rate of the complex. The carboxy terminus of p53 is a target of variety of signals for regulation of p53 DNA-binding activity. Growth suppressor c-Abl has been shown to interact with p53 in response to DNA damage and over-expression of c-Abl leads to G1 growth arrest in a p53-dependent manner. In past budget year, we showed that c-Abl binds directly to the carboxyl terminus of p53 and this interaction requires a tetrameric conformation of p53. In this budget year, we have obtained highly purified c-Abl protein via a baculovirus expression system and shown that c-Abl stimulates dramatically p53's DNA-binding activity in EMSA assay (please see Figure 3 from enclosed ms by Nie et al.). In addition, we have studied mechanisms for c-Abl activation and shown that c-Abl

stimulates p53's DNA-binding activity by decreasing off-rate of the p53-DNA complex (please see Figure 5 from enclosed ms by Nie et al.).

c-Abl stimulates p53's DNA-binding activity in a kinase-independent manner. To study whether c-Abl's kinase activity is involved, we found that addition of a non-hydrolyzable ATP analog, does not prevent c-Abl from stimulating p53's DNA-binding in EMSA (please see Figure 4 from enclosed ms by Nie et al.). Consistent with this, a kinase inactive c-Abl mutant, K(290)R, was also shown to be able to activate p53-mediated transcription like wt c-Abl. Together, our data showed that the c-Abl stimulation does not require its tyrosine kinase activity, suggesting a novel function of c-Abl.

Stimulation of p53-mediated transcription by c-Abl requires carboxyl terminus of p53. If the activation of p53's DNA-binding by c-Abl via carboxyl terminus has functional importance we reasoned that c-Abl should affect p53 transcription activity via carboxyl terminus. We tested this hypothesis by examining the effect of c-Abl on transcription mediated by either p53 or a carboxyl terminal mutant p53, Δ363. Our results show that c-Abl enhances the transcriptional activity of p53, but not Δ363 (please see Figure 6 from enclosed ms by Nie et al.). These finding show a correlation between activation of p53 transcription by c-Abl and ability to stimulate p53's DNA-binding.

CONCLUSIONS

The finding that c-Abl stimulates p53's DNA-binding via the negative regulatory domain may provide important clues about the regulation of p53. It has been suggested by other investigators that peptides designed to interact with this region can be used to reactivate p53 pathway in tumor cells to cause cell cycle arrest. In spite of the significance of this region, however, no growth regulatory protein has been shown to function directly via this region. In the this study, we present results that clearly indicate a link between the growth suppressor c-Abl and p53 transcription regulation, and importantly, provide an example of activation of p53 DNA-binding activity via the carboxyl terminal regulatory domain by a cell-cycle protein.

APPENDED TO THE SUMMARY

Research Accomplishments:

- c-Abl stimulates p53's DNA-binding ability by decreasing off-rate of the complex
- c-Abl stimulates p53's DNA-binding activity in a kinase-independent manner
- Stimulation of p53-mediated transcription by c-Abl requires carboxyl terminus of p53

Reportable Outcomes:

Publications:

Song, X.D., H.M. Sheppard, A.W. Norman and X. Liu. 1999. MAP-kinase is involved in the degradation of p53 protein in the bryostatin-1 induced differentiation of the acute promyelocytic leukemia cell line NB4. *J. Biol. Chem.* **274**:1677-1682.

Sheppard, H.M., S.I. Corneillie, C. Espiritu, A. Gatti and X. Liu. 1999. New insights into the mechanism of inhibition of p53 by SV40 large T antigen. *Mol. Cell. Biol.* **19**:2746-2753.

Nie, Y., C.M. Bula and X. Liu. Stimulation of p53 DNA-binding by c-Abl requires the p53 C-terminus and tetramerization. Submitted.

A copy of each of the above cited ms:

Enclosed

Stimulation of p53 DNA-binding by c-Abl requires the p53 C-terminus and tetramerization

Ying Nie, Craig M. Bula and Xuan Liu[1]

Department of Biochemistry, University of California, Riverside, CA 92521

Running title: c-abl stimulates DNA binding activity of p53

Key words: p53, c-Abl, C-terminal regulatory domain, DNA-binding, tetramerization

[1]Corresponding author. Email: xuan.liu@ucr.edu; FAX (909) 787-4434

ABSTRACT

The carboxyl terminus of p53 is a target of a variety of signals for regulation of p53 DNA-binding. Growth suppressor c-Abl interacts with p53 in response to DNA damage and over-expression of c-Abl leads to G1 growth arrest in a p53-dependent manner. Here, we show that c-Abl binds directly to the carboxyl terminal regulatory domain of p53 and that this interaction requires tetramerization of p53. Importantly, we demonstrate that c-Abl stimulates the DNA-binding activity of wild-type p53 but not of a carboxyl-terminally truncated p53 (p53Δ363C), and furthermore, a deletion mutant of c-Abl that does not bind to p53 is also incapable of activating p53 DNA-binding, suggesting that the binding to the p53 carboxyl terminus is necessary for c-Abl stimulation. To investigate the mechanism for this activation, we have also shown that c-Abl stabilizes the p53-DNA complex. Interestingly, the stimulation of p53 DNA-binding by c-Abl does not require its tyrosine kinase activity, indicating a new function for c-Abl. Together, these observations provide evidence for a role of c-Abl as a growth suppressor protein in activation of p53 DNA-binding via the carboxyl terminal regulatory domain and tetramerization.

INTRODUCTION

p53 exerts its tumor suppression function by inducing growth arrest and apoptosis (Ko and Prives 1996; Levine 1997). The biochemical activity of p53 that is required for this relies on its ability to bind to specific DNA sequences and to function as a transcription factor (Pietenpol et al. 1994). The importance of the activation of transcription by p53 is underscored by the fact that the majority of p53 mutations found in tumors are located within the domain required for sequence-specific DNA binding (Ko and Prives 1996; Levine 1997). Therefore, it is clear that this activity is critical to the role of p53 in tumor suppression.

A contiguous stretch of 30 amino acid residues at the carboxyl terminus of p53 (C-terminus; amino acid 363 to 393) constitutes a domain required for regulation of p53 DNA-binding. Interference with this domain by modification, including phosphorylation (Hupp et al. 1992, for review see Prives 1998) and acetylation (Gu and Roeder 1997; Liu et al. 1999), or by deletion (Hupp et al. 1992) has been shown to enhance p53 DNA-binding activity. Moreover, several proteins, including Ref-1 (Jayaraman et al. 1997) and 14-3-3 (Waterman et al, 1998), have been shown to bind to this region of p53 and enhance the DNA binding activity of p53. These studies defined the C-terminal domain as a negative regulatory domain which normally results in a latent, low-affinity DNA-binding form of p53. Therefore, it follows that, upon DNA damage, signals which regulate cell growth may also function through this domain to stimulate p53 DNA-binding.

Recently, the c-Abl tyrosine kinase has been shown to interact with p53 in response to DNA damage (Yuan et al. 1996a; 1996b; Kharbanda et al, 1998) and over-expression of c-Abl leads to G1 growth arrest in a p53-dependent manner (Goga et al. 1995). In addition, c-Abl was also found to enhance the ability of p53 to activate transcription from a promoter containing a p53 DNA-binding site in transient transfection assays (Goga et al. 1995) and to stimulate the expression of p21 (Yuan et al. 1996b). Deletion of the p53 binding domain in c-Abl (ΔProl, a deletion of a proline rich domain, amino acid 793-1044) impairs the ability of c-Abl to stimulate p53 transcriptional activity and to suppress growth (Goga et al. 1995). These results suggest that

the p53-Abl interaction plays an important role in regulation of p53-mediated transcription and growth suppression. It is important to note, however, that a kinase-inactive form of c-Abl [c-Abl(K-R)], which is defective in its ability to suppress growth, was also found to enhance the ability of p53 to activate transcription (Goga et al. 1995; Yuan et al. 1996b), suggesting that the tyrosine kinase activity of c-Abl is required for growth suppression but not for transcriptional activation. These data argue that c-Abl may stimulate p53-dependent transcription in a kinase-independent manner. Consequently, a detailed understanding of how c-Abl stimulates p53-dependent transcription is of significance.

In the present study, we show that c-Abl binds directly to the C-terminus of p53 and this interaction requires a tetrameric conformation of p53. Furthermore we demonstrate that c-Abl significantly stimulates the DNA-binding of p53 but not of a C-terminally truncated form of p53 (p53Δ363C), suggesting that the interaction with the p53 C-terminus is necessary for c-Abl stimulation. To determine the mechanism for this activation, we have shown that c-Abl stabilizes the p53-DNA complex. As discussed above, the tyrosine kinase activity of c-Abl is not required for p53 transcriptional activation. Consistent with this, we show that the stimulation of p53 DNA-binding by c-Abl does not require its tyrosine kinase activity, indicating a novel function of c-Abl. Together, these observations indicate a mechanism of activation via the p53 C-terminal regulatory domain by the growth suppressor protein c-Abl.

MATERIALS AND METHODS

Plasmid construction

The p53Δ292 deletion mutant was constructed by PCR amplification of amino acid 1-292 of p53 from pcDNA-p53 (Liu et al. 1993), using primers that introduce Hind III sites at both 5' and 3' ends. The amplified DNA fragments were then cloned into the Hind III site of pcDNA.A (Invitrogen). The p53Δ363 deletion mutant was constructed by PCR amplification of amino acid 1-363 of p53 from pcDNA-p53, using primers that introduce a Hind III site at the 5' end, and an Eco RI site and stop codon at the 3' end. The amplified DNA fragments were then cloned into the Hind III and Eco RI sites of pcDNA. The internal deletion mutants of p53, p53Δ325-356 and p53Δ316-322, were generated from pcDNA-p53 by PCR using a pair of inverted primers that would delete the base pairs coding for the corresponding amino acids. PCR products were then phosphorylated with T4 Kinase and ligated. Similarly, the p53ΔTet mutant (341K344E348E355K), according to Stürzbecher, et al. (1992), was constructed using primers that contained mutations at corresponding amino acid positions. All mutant constructions were confirmed by sequencing analysis. The luciferase reporter plasmid, pRGCE4Luc, was constructed by cloning the BamHI-Asp718 fragment containing RGCE4TATA from pRGCE4CAT (Goga et al. 1995) into pZLuc (Sladek et al. 1998).

Purification of c-Abl and p53 proteins

Sf21 cells were infected with recombinant baculoviruses expressing GST-Abl, GST-Abl-ΔSH3 (a deletion construct of c-Abl lacking the SH3 domain; Mayer and Baltimore, 1994) and GST-Abl-ΔC (a deletion construct of c-Abl truncated after the catalytic domain, amino acid 532; a gift from Dr. B. Mayer, Harvard University) and lysed as described by Pendergast *et al.* (1991). The GST fusion proteins were then bound to Glutathione-Sepharose (Pharmacia), and eluted with buffer containing 10 mM glutathione, and purified protein were dialyzed in 0.1 M KCl D buffer (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). To purify p53, HeLa cells were infected with recombinant

vaccinia virus expressing an epitope-tagged p53 (HA-p53) and p53 was either purified from the nuclear extract of infected cells by binding to matrix of monoclonal antibody (12CA5) specific for the epitope tag, followed by elution with the epitope peptide as described by Liu and Berk (1995), or purified with a matrix of pAb 421 anti-p53 as described by Sheppard et al. (1999). The purified proteins were analyzed by SDS-PAGE. To purify p53Δ363, Sf21 cells were infected with recombinant baculovirus expressing HA-p53Δ363 (a gift from Dr. C. Prives, Columbia University), and p53Δ363 was purified with 12CA5 antibody as described above.

GST pull-down assay

Wild type and mutant p53 RNAs were synthesized according to conditions recommended by the manufacturer (Promega). The mRNAs were translated in vitro for 1.5 hr at 30°C in rabbit reticulocyte lysate in the presence of ^{35}S-methionine. Bacterially expressed GST-Abl-C (a portion of the c-Abl carboxyl-terminus starting at amino acid 711) has been shown to bind to p53 (Goga and Liu, unpublished data). Glutathione-Sepharose (Pharmacia) was washed thoroughly in lysis buffer (10% glycerol, 1% Triton X-100, 10mM EDTA in PBS) and incubated for 60 min with GST fusion protein. Beads were then incubated for 60 min with the ^{35}S-methionine labeled p53 mutants in incubation buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM PMSF, 50 μg/ml ethidium bromide, 10 μg/ml aprotinin and 10 μg/ml leupeptin) with constant mixing. After incubation, the beads were washed three times with incubation buffer and boiled in 15 μl of 2x SDS sample buffer. The bound proteins were analyzed by SDS-PAGE and ^{35}S-labeled proteins were visualized by autoradiography.

EMSA

EMSA was carried out as described (Sheppard et al. 1999). Briefly, The RGC p53-binding site probe (5'- AGCTTGCCTCGAGCTTGCCTGGACTTGCCTGGTCGACGC -3') was labeled with the Klenow fragment of *E. coli* DNA polymerase. Binding reactions contained 60 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 1 μg BSA, 0.1 μg poly [d(GC)], 200 cpm of ^{32}P-labeled

probe, proteins as indicated, in a total volume of 12.5 µl. Reactions were incubated at 30°C for 40 min or as indicated when association experiments (on-rate) were performed, and then analyzed on a 5% polyacrylamide gel containing 0.5 x TBE (0.045 mM Tris-borate, 0.045 mM sodium borate, 0.001 mM EDTA [pH 8.0]). DNA-protein complexes were visualized with a PhosphorImager using Adobe Photoshop software. When required, reactions were incubated in the presence of 2 mM ATPγS, a nonhydrolyzable ATP analog, to inhibit kinase activity. When dissociation experiments (off-rate) were performed, reactions were incubated for 40 min immediately followed by an addition of 20X excess of unlabeled RGC oligonucleotide to challenge the DNA-protein complex for indicated times.

Transcriptional activation assay

The transcription activity of p53 was measured using pRGCE4Luc which contains one copy of the RGC p53 binding site cloned upstream of the adenovirus E4 TATA box and luciferase gene. Various combinations of the plasmid DNAs listed in Figure 6 were transfected into Saos-2 cells using calcium phosphate. The amounts of plasmids transfected for 60 mm plates were as follows: 0.5 µg of pRGCE4Luc, 0.2 µg of pcDNA-p53, 0.2 µg of pcDNA-p53Δ363, and 0.5 µg of pSRαMSVtkNeo-Abl (Goga et al. 1995). All samples for luciferase assays were normalized for β-galactosidase activity from a co-transfected control expression vector as described (Liu et al. 1993).

RESULTS

c-Abl interacts with the C-terminal regulatory domain of p53. It has been shown previously that c-Abl binds to p53 and activates p53-dependent transcription. To investigate the mechanism by which c-Abl stimulates transcription, we mapped the domains on p53 that are required for c-Abl binding, as p53 can be regulated via different mechanisms through protein-protein interaction at different functional domains. A panel of N- or C-terminus truncated p53 mutants (Figure 1A) were in vitro translated in the presence of ^{35}S-methionine and incubated with immobilized GST and GST-Abl-C which contains p53 binding domain as reported by Goga et al (1995). After incubation, the beads were washed and proteins bound to the beads were analyzed by SDS-PAGE (Figure 1B). Deletion of the p53 transactivation domain (p53Δ92) had no effect on binding to GST-Abl. In contrast, deletion of the p53 carboxyl-terminus (p53Δ292C) completely abrogated binding to GST-Abl-C, suggesting the carboxyl-terminal region is required for c-Abl binding.

The C-terminus (amino acid 293 and 393) harbors functional domains responsible for nuclear localization (amino acid 316-322), tetramer formation (amino acid 325-356) and regulation of p53 DNA-binding (amino acid 363-393). To further localize the c-Abl binding domain within the C-terminus of p53, we next conducted GST-Abl binding assays with a series of p53 C-terminal small deletion mutants (p53Δ316-322, p53Δ325-356 and p53Δ363C; Figure 1A) in which each of these three functional domains was individually removed. Because c-Abl and p53 are both DNA binding proteins, we included 50 μg/ml of ethidium bromide to the binding buffer to disrupt possible interactions mediated through protein-DNA interactions. Figure 1C shows the GST binding results. Deletion of the p53 nuclear localization signal (p53Δ316-322) had no effect on binding to c-Abl. However, deletion of the regulatory domain in p53, p53Δ363C significantly disrupted its ability to bind to c-Abl. This region has been previously identified as an inhibitory domain for p53 DNA-binding. Furthermore, deletion of the tetramerization domain, Δ325-356, also greatly reduced binding to c-Abl. These findings demonstrate that the interaction between c-Abl and p53 requires the C-terminal regulatory domain and tetramerization domain of p53.

Tetrameric conformation is necessary for the p53-cAbl interaction. The tetrameric domain is important for higher order p53 complex formation, DNA binding, phosphorylation at Ser15, Ser20 and Ser33 as well as the dominant negative effect of mutant p53 molecules over wild-type p53 (Unger et al, 1993; Shieh et al. 1999). The results from the GST binding experiments in Figure 1 led us to hypothesize that c-Abl interacts with the regulatory domain of p53 and that this interaction may require the tetrameric conformation of p53. It is also possible, however, that c-Abl may interact with residues in the tetramer domain directly. To test this, we constructed a tetramerization impaired mutant, 341K344E348E355K (Tet Mut), which contains four mutated residues at positions 341, 344, 348 and 355 as reported by Stürzbecher, et al, (1992). The ability of the mutant to interact with c-Abl was tested using the GST pull-down assay as described above (Figure 2). The results showed that this mutant, similar to Δ325-356, fails to bind to c-Abl, revealing the requirement of the tetrameric conformation of p53 for c-Abl interaction.

Activation of p53-DNA binding by c-Abl requires p53 C-terminus. If the interaction of c-Abl with the regulatory domain of p53 is of functional significance, we reasoned that c-Abl should alter the negative regulatory effect of the C-terminus on p53 DNA-binding. To test this possibility, we examined the effect of c-Abl on p53 DNA-binding in EMSA with a probe containing the p53 *cis* element identified in the RGC as described by Sheppard et al (1999). As expected, 50 ng of vaccinia virus-expressed epitope-tagged human p53 (one-half the amount as shown in Figure 3B, lane 2) purified from HeLa cells with 12CA5 antibody bound to this probe and produced a retarded p53-DNA complex (Figure 3A, lane 2). Addition of 30 ng of baculovirus expressed GST-Abl (one tenth of the amount shown in Figure 3B, lane 3; ~1:1 molar ratio of c-Abl/p53 tetramer) resulted in a marked stimulation of the p53 DNA-binding (5 to 10-fold activation, Figure 3A, lanes 2 and 6). This c-Abl-stimulated p53-DNA complex, however, could not be supershifted with anti-Abl antibody, suggesting that c-Abl was not associated with the p53-

DNA complex (data not shown). In contrast to c-Abl, the same amount of control extract purified from mock infected cells (C; Figure 3A, lane 3), GST protein (G; lane 4) or a combination of both (C\G; lane 5) were incapable of stimulating p53 DNA-binding. Each of the extracts used in this experiment have been tested over a range of concentrations corresponding from one-half to four times the amount used in Figure 3A. At each of the concentrations tested, stimulation by c-Abl was observed (data not shown). These results suggest that c-Abl interacts with the p53 regulatory domain to relieve its negative effect on p53 DNA-binding. The fact that stoichiometric quantities of c-Abl stimulates p53 DNA binding support the view that tetrameric conformation of p53 is necessary for the stimulation.

If the c-Abl interaction plays a role in the activation of p53 DNA-binding, we reasoned that a construct of c-Abl, that lacks the p53 binding domain (c-Abl-ΔC), should not activate p53 DNA-binding, whereas another construct of c-Abl, that binds to p53 but lacks SH3 domain (c-Abl-ΔSH3), should activate p53 DNA-binding. We tested this hypothesis by comparing the ability of wild-type c-Abl, c-Abl-ΔSH3 and c-Abl-ΔC to stimulate p53 DNA-binding (Figure 3C). The c-Abl-ΔSH3 mutant continued to activate p53 DNA-binding whereas c-Abl-ΔC was significantly impaired in its ability to stimulate p53 DNA-binding. These findings demonstrate a correlation between the ability to bind p53 and to activate p53 DNA-binding. Consequently, c-Abl interaction is required for activation of p53 DNA-binding.

If the results are correct, c-Abl should not affect the DNA-binding of the C-terminal truncated form of p53 (Δ363C). To test this hypothesis, we performed a gel-shift assay with Δ363C in the presence of c-Abl or a control GST extract (Figure 3D). As expected, p53 resulted in a slowly migrating band, the p53-DNA complex (lane 2), and addition of c-Abl significantly activated the p53 DNA-binding (lane 3). In contrast, Δ363C bound to DNA far more efficiently than p53 (compare lane 4 to lane 2). Addition of c-Abl to Δ363C, however, did not have any effect on DNA-binding (lanes 5 and 7). The striking difference in activation of p53 DNA-binding by c-Abl supports our conclusion that c-Abl interacts with the regulatory domain to diminish its negative regulatory effect on p53 DNA-binding.

It has been demonstrated previously that 421 antibody binds to the regulatory domain (amino acid 372 to 382) and activates p53 in a manner similar to that observed with c-Abl. Therefore, we tested whether c-Abl can stimulate p53 purified with 421 antibody. Surprisingly, 421-purified p53 can be activated by c-Abl to a similar extent as 12CA5-purified p53 (Figure 3A, lanes 7 to 9). Several explanations may account for this result. c-Abl and 421 antibody may bind to different regions on the negative regulatory domain and affect p53 DNA-binding independently. Support for this mechanism comes from the interaction data in that c-Abl binding, unlike 421 antibody, requires the tetrameric conformation of p53 (Figure 2). Alternatively, c-Abl may alter the conformation of the regulatory domain more efficiently, resulting in a further stimulation of DNA-binding of 421-purified p53.

c-Abl activates p53-DNA binding in a kinase-independent manner. Because a kinase-inactive form of c-Abl [c-Abl(K-R)], has been reported to bind p53 and enhance the ability of p53 to activate transcription (Goga et al. 1995; Yuan et al. 1996b), we considered that the kinase activity of c-Abl might not be required for the activation of p53 DNA-binding. We therefore examined the effect of ATPγS, a non-hydrolyzable ATP analog that inhibits c-Abl kinase activity in GST-Crk phosphorylation and autophosphorylation (data not shown) on the ability of c-Abl to stimulate p53 DNA-binding. Figure 4 shows that addition of 2 mM of ATPγS did not have any effect on the activation of p53 DNA-binding by c-Abl (compare lane 4 to lane 2). As a control, ATPγS was also added to p53 DNA binding reaction without c-Abl and no effect on p53 DNA-binding was observed (compare lane 7 to lane 5). These results suggest that the activation of p53 DNA-binding by c-Abl is kinase independent. These results are consistent with the previous observation that c-Abl enhances the ability of p53 to activate transcription in a kinase-independent manner and suggest that c-Abl activation of p53 DNA-binding occurs independent of its function as a protein tyrosine kinase.

Interaction with c-Abl stabilizes the p53-DNA complex. The activation of p53 DNA-binding by c-Abl could result from increasing the rate of p53-DNA complex formation or by decreasing the rate at which p53 dissociates from the DNA.

To determine whether c-Abl affects the rate of p53-DNA complex formation, p53 was incubated with the RGC probe in the presence or absence of c-Abl or the GST control. At different time points (0, 2, 5, 10, 20, 40 and 60 min) after mixing, aliquots of the reaction mixture were loaded on a running gel (Figure 5A). The results show that p53 gel-shift bands were observed at maximal levels 2 min after incubation, in the presence or absence of c-Abl, suggesting that formation of the p53-DNA complex may not be affected by the presence of c-Abl. Of note, a decreased level of p53-DNA complex was observed after 40 min incubation without c-Abl, but not with c-Abl, indicating that c-Abl may stabilize p53-DNA complex.

To test this hypothesis, we next determined whether c-Abl decreased the dissociation rate of a pre-formed p53-DNA complex. For this experiment, purified p53, plus or minus c-Abl, was incubated with the RGC probe for 30 min, and then the formed complexes were challenged with a 20-fold molar excess of unlabeled RGC oligonucleotide as a competitor. Aliquots of the reaction mixture were loaded on a running gel at 0, 5, 10, 20, 40 and 60 min after the addition of the competitor DNA (Figure 5B). At the end of the electrophoresis, the amount of DNA shifted was quantitated by PhosphorImager analysis. Figure 5C shows a plot of the data from four independent experiments where 100% represented the amount of p53-DNA complex formed before the addition of unlabeled competitor (0 min). The data clearly show that c-Abl stabilizes the p53-DNA binding.

The C-terminus of p53 is responsible for activation of p53-dependent transcription by c-Abl. Our results showing that c-Abl interacts with the C-terminal regulatory domain to activate p53 DNA-binding, prompted us to determine the effect of c-Abl on the ability of p53 and Δ363C to activate transcription *in vivo*. Toward this end, we performed transient transfection experiments from a minimal promoter containing one p53 binding site (RGC) and E4 TATA box (RGCE4) in Saos-2 cells. Figure 6 summarizes the results from three independent

experiments in which RGCE4 was cotransfected with expression vectors for p53 or Δ363C, with or without c-Abl, and luciferase was measured after adjusting for the difference in transfection efficiency. As expected, addition of c-Abl to full-length p53 resulted in a two and half-fold enhancement of activation. In contrast, addition of c-Abl to Δ363C had no detectable effect on the transcription. The enhancement by c-Abl is p53-dependent as c-Abl alone over a wide range had no effect on the RGCE4 promoter (data not shown). These findings support our conclusion that the C-terminus of p53 is responsible for activation of p53-dependent transcription by c-Abl.

DISCUSSION

A mechanism for c-Abl activation of p53-dependent transcription

Although it is known that c-Abl stimulates p53-dependent transcription, a function required for c-Abl growth suppressor activity (Goga et al, 1995), the molecular mechanism by which c-Abl stimulates p53-dependent transcription is still largely unclear. The results reported here show that c-Abl interacts with the C-terminal regulatory domain of p53 in a tetramerization-dependent manner and functions to activate the p53 DNA-binding. In an effort to assess the mechanism of c-Abl activation, we also show that c-Abl activates p53 DNA-binding by stabilizing the p53-DNA complex. Collectively, these results suggest a model for c-Abl activation. In this model c-Abl activates latent p53 by relieving the C-terminal inhibitory domain of p53 and enhances p53 DNA-binding by forming a stable p53-DNA complex. Support for this mechanism also comes from the correlation between the effect of c-Abl mutations on the interaction with p53 and on the activation of p53 DNA-binding. These results indicate that c-Abl contributes to p53 transactivation by functioning as a stimulator of p53 DNA-binding.

c-Abl functions as a p53 DNA-binding stimulator in a manner different from several other stimulator proteins which also activate DNA-binding by relieving the C-terminal inhibitory effect. Examples include p300, which acetylates lysine residues at the C-terminus and activates latent p53 (Gu and Roeder, 1997) and Ref-1, which activates p53 DNA-binding via the C-terminal domain in a redox-dependent manner. c-Abl is distinct from these proteins in that it did not appear to covalently modify p53 or to rely on the redox state of p53. It may be similar in this regard to 421 antibody activation which binds to the C-terminal domain and relieves its negative effect on p53 DNA-binding. However, 421 antibody recognizes both tetrameric and monomeric forms of p53, indicating a conformation-independent binding. In the case of c-Abl, the specific binding of c-Abl requires p53 to be in a tetrameric form. What remains to be determined is the significance of c-Abl binding the p53 tetramer but not the monomer.

Our data suggest that c-Abl stimulates p53-mediated transcription, at least in part, by activation of p53 DNA-binding. Of note, two recent studies have shown that overexpression of c-

Abl also induces p53 accumulation (Yuan et al 1996b) probably via the neutralization of the inhibitory effect of mdm2 by c-Abl (Sionov et al, 1999). These data suggest that the c-Abl-p53 interaction induces a conformational change which may dissociate p53 from Mdm2. p300 has been shown to activate p53 via two different mechanisms, activation of p53 DNA-binding (Gu and Roeder, 1997; Liu et al, 1999) and stabilization of the p53 protein (Yuan et al, 1999). Therefore, similar to the activation of p53 by p300, our data together with the studies cited above suggest that c-Abl may stimulate p53-mediated transcription by more than one mechanism, i.e. enhanced DNA binding as well as protein accumulation.

Proposed model for the stabilization of the p53-DNA complex by c-Abl

We have shown that c-Abl stabilizes the p53-DNA complex. In order to explain this increased stability, we speculate that the interaction of c-Abl with the C-terminus of p53 may stabilize the p53 tetramer. There are several reasons which led us to believe this is the case. Our results show that c-Abl interacts with the tetrameric form of p53, but not with the monomeric form, suggesting that multiple contacts between c-Abl and p53 may be required for the interaction. These multiple contacts, in principle, could induce a stable tetrameric form of p53, resulting in a more stable protein-DNA complex (McLure and Lee, 1998). Of note, it has been suggested that dimerization of nuclear receptors stabilizes the binding of the receptors to DNA (Glass et al. 1994). In the absence of the dimerization domain, DNA binding domain (DBD) monomers, dissociate from the DNA very rapidly. In contrast, a dimer of the full-length receptor was found to dissociated from the DNA very slowly. These findings were explained in a one-step/two-step model by Jiang et al. (1997) in which the DBD monomers dissociated from the DNA one at a time in two energetically favorable steps, whereas the full-length receptor dissociated from the DNA in a single step process. This one-step dissociation was considered to be energetically unfavorable, since the contacts between two DBD monomers and DNA had to be broken at the same time. This model could also apply to other DNA-binding proteins such as p53. In this case, the stabilization of DNA binding would be more effective as p53 exists as a tetramer.

It is tempting to speculate that our hypothesis may also explain why the C-terminal domain inhibits p53 DNA-binding. The C-terminal regulatory domain was proposed to interact with a motif in the core of the p53 tetramer, thereby forming a conformationally inactive complex (Hupp et al. 1995). An alternative explanation, however, is that the C-terminal domain may interfere with the tetramerization of p53, resulting in a less stable p53-DNA complex. Supporting this assumption is the experimental evidence that Δ363C also stabilizes the p53-DNA complex (data not shown). The fact that the C-terminal domain is closely located next to the tetramerization domain also makes this alternative model physically possible. Further studies of the role of the C-terminal regulatory domain in tetramerization will be required to distinguish between these possibilities.

A kinase-independent activity for c-Abl

The c-Abl protein is a nuclear tyrosine kinase. However, c-Abl-p53 complexes are detected in cells expressing either wild-type or the kinase-inactive c-Abl (K-R) in response to ionizing radiation (Yuan et al, 1996b). Furthermore, the kinase activity of c-Abl is not required for transcriptional activation by p53 in transient transfection assays from a promoter containing p53 DNA-binding sites (Goga et al 1995). Consistent with these results, our data reveal that the c-Abl kinase activity is not required for the activation of p53 DNA-binding. On the basis of these observations, we propose a kinase-independent activity for c-Abl: activation of p53 DNA-binding. Several lines of evidence have lent support to this activity. First, although a deletion of the c-Abl SH3 domain increases Abl-mediated tyrosine phosphorylation in vivo (Franz et al, 1989; Jackson and Baltimore, 1989; Van Etten et al, 1995), similar effects on transactivation (Goga et al, 1995) by wild-type Abl and Abl-ΔSH3 (a deletion of c-Abl lacking SH3 domain) were observed. Second, the amounts by which the kinase-inactive c-Abl(K-R) stabilizes p53 were similar to the stabilization by wild-type c-Abl (Sionov et al, 1999), suggesting that c-Abl functions to induce a conformation change in p53 in a non kinase-dependent manner. Finally, the overexpression of both wild-type c-Abl and c-Abl(K-R) enhances the expression of endogenous p21 (Yuan et al, 1996b).

A link between DNA-damage and activation of p53 via the C-terminal domain

c-Abl contributes to radiation-induced G1 arrest via a p53-dependent mechanism (Yuan et al, 1996b), indicating that p53 lies in a pathway downstream from c-Abl. We demonstrate that c-Abl binds to the C-terminus of p53 and stimulates p53 DNA-binding. These findings directly link c-Abl to activation of p53 DNA-binding via the C-terminal domain in response to DNA-damage. Interestingly, a recent study has shown that IR leads to dephosphorylation of Ser376, resulting in an association of 14-3-3 proteins with p53 via the C-terminal domain which, in turn, enhanced the affinity of p53 for sequence-specific DNA (Waterman et al, 1998). This observation suggests that p53 lies in a pathway downstream from the 14-3-3 protein in response to DNA-damage. Our data together with this finding support the view that there are multiple molecular pathways that signal DNA damage (Liu et al, 1996) and activate p53 via the C-terminal domain. Although it is clear that the interaction of p53 with c-Abl is DNA damage-inducible, it remains to be determined whether Ser376 dephosphorylation contributes to such a c-Abl-p53 association.

Transient transfection assays showed a significant stimulatory effect of c-Abl on the ability of cotransfected p53 to activate transactivation. The observation that c-Abl did not stimulate p53Δ363C in these assays supports the assumption that the C-terminal domain of p53 is likely targeted by DNA-damage signaling pathways *in vivo*. Definitive evidence for a loss of c-Abl response in cells expressing p53Δ363C will be required to validate such a model. In addition, further analysis of the effects of c-Abl on the promoters of natural p53 response genes such as p21 in such cells should help to clarify this issue.

The function of p53 in the DNA damage response is clearly important to the proper functioning of many cell types. In this study, we have provided an example of activation of p53 DNA-binding via the C-terminal regulatory domain by a growth suppressor protein, c-Abl. Our finding further supports the view that the C-terminus of p53 is a target for stimulation of p53 DNA-binding in response to DNA damage and suggests that tetramerization is required for the stimulation.

ACKNOWLEDGMENTS

We are grateful to Dr. B. Mayer (Harvard University) for providing baculoviruses expressing GST-cAbl, GST-cAbl–ΔSH3, particular GST-cAbl–ΔC prior to publication; and to Dr. C. Prives (Columbus University) for baculovirus expressing p53Δ363C. We wish to thank Drs. F. Sladek, A. Goga, N. L'Etoile, A. Merino and S. Corneillie for many helpful discussion and valuable comments on the manuscript. This work was supported by grants CA75180 (X.L.) from the National Cancer Institute and DAMD17-96-6076 (X.L.) from the U.S. Army Breast Cancer Research Program.

REFERENCES

Franz, W.M., P. Berger, and J.Y.J. Wang. 1989. EMBO J. **8**:137-147.

Glass, C.K. 1994. Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr. Rev. **15**:391-407.

Goga, A., X. Liu, T.M. Hambuch, K. Senechal, E. Mayor, A.J. Berk, O.N. Witte, and C.L. Sawyers. 1995. p53-dependent growth suppression by the c-Abl nuclear tyrosine kinase. Oncogen **11**:791-799.

Gu, W., and R.G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell **90**:595-606

Hupp, T.R, D.W. Meek, C.A. Midgley, and D.P. Lane. 1992. Regulation of the specific DNA binding function of p53. Cell **71:**875-886.

Jackson and Baltimore. 1989.

Jayaraman, L., K.G.K. Murthy, C. Zhu, T. Curran, S. Xanthoudakis, and C. Prives. 1997. Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes Dev. **11:** 558-570.

Jiang, G., U. Lee and F.M. Sladek. 1997. Proposed Mecanism for the stabilization of nuclear receptor DNA binding via protein dimerization. Mol. Cell Biol. **17:**6546-6554.

Kharbanda, S., Z.M. Yuan, R. Weichselbaum, and D. Kufe. 1998. Determination of cell fate by c-Abl activation in the response to DNA damage. Oncogene **17**:3309-3318.

Ko, L.J., and C. Prives. 1996. p53: Puzzle and paradigm. Genes Dev. **10**:1054-72.

Levine, A.J. 1997. p53, the cellular gatekeeper for the growth and division. Cell **88**:323-331.

Liu, X., C.W. Miller, H.P. Koeffler, and A.J. Berk. 1993. The p53 activation domain binds the TATA box-binding polypeptide in holo-TFIID, and a neighboring p53 domain inhibits transcription. Mol. Cell Biol. **13:**3291-3300.

Liu, X., and A.J. Berk. 1995. Reversal of *in vitro* p53 squelching by both TFIIB and TFIID. Mol. Cell Biol. **15:**6474-6478.

Liu, Z.G., R. Baskaran, E.T. Lea-Chou, L.D. Wood, Y. Chen. M. Karin, and J.Y.J. Wang. 1996. Three distinct signalling responses by murine fibroblasts to genotoxic stress. Nature **384**:273-276.

Liu, L., D.M. Scolnick, R.C. Trievel, H.B. Zhang, R. Marmorstein, T.D. Halazonetis, and S.L. Berger. 1999. p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. Mol. Cell Biol. **19:**1202-1209.

Mayer, B.J., and D. Baltimore. 1994. Mutagenic analysis of the roles of SH2 and SH3 domains in regulation of the Abl tyrosine kinase. Mol. Cell Biol. **14**:2883-94.

McLure, K.G., and P.W. Lee. 1998. How p53 binds DNA as a tetramer. EMBO J. **17**:3342-3350.

Pendergast, A.M., A.J. Muller, M.H. Havlik, Y. Maru, and O.N. Witte. 1991. BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL Sh2 regulatory domain in a non-phosphotyrosine-dependent manner. Cell **66**:161-171.

Pietenpol, J.A., T. Tokino, S. Thiagalingam, W.S. Ei-Deiry, K.W. Kinzler, and B.S. Vogelstein. 1994. Proc.Nat.Acad.Sci. **91**:1998-2002

Prives, C. 1998. Signaling to p53: breaking the Mdm2-p53 circuit. Cell **95**:5-8.

Sheppard, H.M., S.I. Corneillie, C. Espiritu, and X. Liu. 1999. New insight into the mechanism of inhibition of p53 by Simian Virus 40 large T antigen. Mol. Cell Biol. **19:**2746-2753.

Shieh, S.-Y., Y. Taya and C. Prives. 1999. DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. EMBO J. **18**:1815-1823.

Sionov, R.V., E. Moallem, M. Berger, A. Kazaz, O. Gerlitz, Y. Ben-Neriah, M. Oren, and Y. Haupt. 1999. C-Abl neutralizes the inhibitory effect of Mdm2 on p53. J. Biol. Chem. **274**:8371-8374.

Sladek, F.M., D.-Y. Qing, and L. Nepomuceno. 1998. MODY1 mutation Q268X in hepatocyte nuclear factor 4a allows for dimerization in solution but cause abnormal subcellular localization. Diabetes **47**:985-990.

Stürzbecher, H.W., B.R. Addison, C. Rudge, K. Remm, M. Grimaldi, M. Keenan, E. Jenkins, JR. 1992. A C-terminal alpha-helix plus basic region motif is the major structural determinant ofp53 tetramerization. Oncogene **7**:1513-23.

Unger, T., J.A. Mietz, M. Scheffner, C.L. Yee and P. Howley. 1993. Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition, and transformation suppression. Mol. Cell. Biol. **13**:5186-5194.

Van Etten, R.A., J. Debnath, H. Zhou, and J.M. Casasnovas. 1995. Oncogene **10**:1977-1988.

Waterman, M.J.F., E.S. Stavridi, J.L.F. Waterman and T.D. Halazonetis. 1998. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. Nature Genetics **19**:175-178.

Yuan, Z.M., Y. Huang, M.M. Fan, C.L. Sawyers, S. Kharbanda, and D. Kufe. 1996a. Genotoxic drugs induce interaction of the c-Abl tryosine kinase and the tumor suppressor protein p53. J. Biol. Chem. **271**:26457-26460.

Yuan, Z.M., Y. Huang, Y Whang, C.L. Sawyers, R. Weichselbaum, S. Kharbanda, and D. Kufe. 1996b. Role for c-Abl tyrosine kinase in growth arrest response to DNA damage. Nature **382**:272-274.

Yuan, Z.M., Y. Huang, T. Ishiko, S. Nakada, T. Utsugisawa, H. Shioya, R. Weichselbaum, Y. Shi, and D. Kufe. 1999. Role for p300 in stabilization of p53 in the response to DNA damage. J. Biol. Chem. **274**:1883-1886.

FIGURE LEGEND

Figure 1. The C-terminal region of p53 is required for association with c-Abl. A. p53 proteins containing N-terminal and C-terminal deletions used in this study. B and C. The p53 protein shown in panel A were translated in vitro and tested for binding to GST-Abl and GST. Binding of the p53 proteins to c-Abl was measured by incubated with immobilized GST-Abl protein, washing, SDS-PAGE and autoradiography of proteins retained on the beads. Binding of p53 protein to GST was measured by incubated with immobilized GST protein in the same condition.

Figure 2. The tetrameric conformation of p53 is necessary for the p53-Abl interaction. Radiolabeled p53 proteins were prepared by in vitro translation and were incubated with either GST or GST-Abl. After washing, proteins were subjected to SDS-PAGE. The tetramerization impaired mutant 341K344E348E355K (Tet Mut) was deficient in binding to c-Abl.

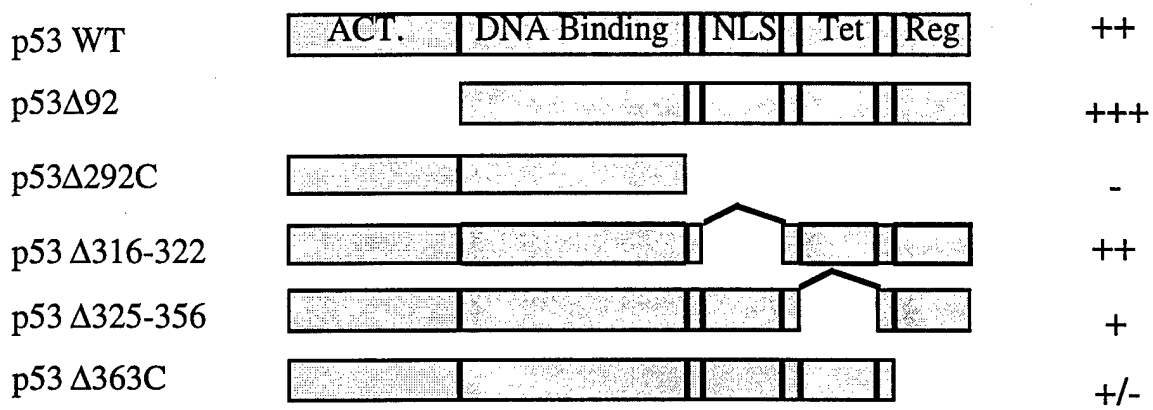
Figure 3. The C-terminal domain is required for c-Abl activation. A. A radiolabeled probe containing the p53-binding site from RGC was incubated with either 50 ng of p53 purified with 12CA5 or 25 ng of p53 purified with 421 in the presence of 30 ng of control extract (C), GST (G) or GST-Abl (A) as indicated. B. A silver-stained SDS-10% PAGE is shown. Lane 1 represents 2 μl of p53Δ363C eluted with HA peptide from a mAb 12CA5 affinity column; lane 2, 2 μl of p53; lane 3, 3 μl of GST-Abl, lanes 4 and 5, 100 and 50 ng of BSA. The sizes (in kD) of molecular mass standards are indicated on the left. C. The RGC probe was incubated with 50 ng of 12CA5 purified p53 in the absence (-) or presence of 30 ng of GST-Abl (A), GST-Abl-ΔC (ΔC) or GST-Abl-ΔSH3 (ΔSH3), as indicated. D. The RGC probe was incubated with 50 ng of 12CA5 purified p53 or 10 ng of 12CA5 purified p53Δ363C in the presence of GST-Abl (A: 30 ng; and 2A: 60 ng) or control extract (C and 2C), as indicated.

Figure 4. c-Abl stimulates p53 DNA-binding in an ATP-independent manner. A radiolabeled probe containing the p53-binding site from RGC was incubated with 50 ng of 12CA5 purified p53 with or without 50 ng of GST-Abl, and with or without 2 mM ATPγS, as indicated.

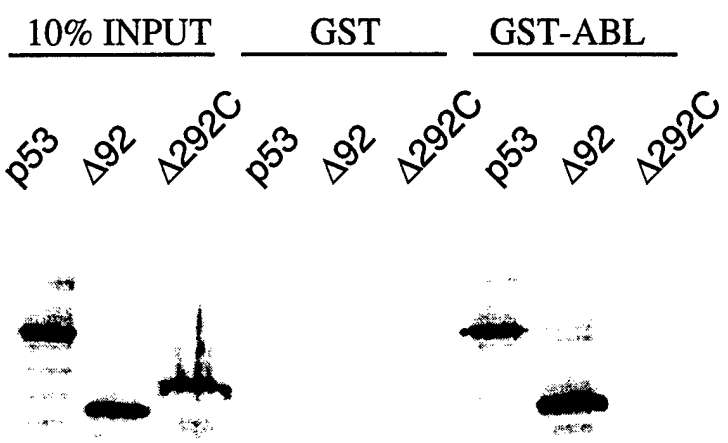
Figure 5. c-Abl prevents the dissociation of the p53-DNA complex. A. Determination of the association rate of the p53-DNA complex in the presence and absence of c-Abl. Binding reactions were performed as described and samples were loaded on a running gel at different time points. B. Determination of the dissociation rate of the p53-DNA complex in the presence and absence of c-Abl. At equilibrum, DNA binding reaction mixtures were challenged by addition of a 20x excess of unlabeled RGC competitor and samples were removed and loaded on a running gel at various time points. C. The intensity of the bands representing the p53-DNA complex in panel B was quantitated with a PhosphorImager and plotted as a percentage of the intensity at equilibrum.

Figure 6. Stimulation of p53 transcriptional activity by c-Abl requires the carboxyl terminus of p53. Saos-2 cells were transfected with the plasmid combination listed below the figure. Luciferase activity was measured after normalization to β-galactosidase activity and expressed as fold of activation relative to the level seen with the reporter alone (lane1). The mean and standard deviations from 3 independent experiments are presented.

A


Binding to cAbl
p53 WT
ACT.
DNA Binding
NLS
Tet
Reg
++
p53Δ92
+++
p53Δ292C
-
p53 Δ316-322
++
p53 Δ325-356
+
p53 Δ363C
+/-

B


10% INPUT
GST
GST-ABL
p53
Δ92
Δ292C
p53
Δ92
Δ292C
p53
Δ92
Δ292C

C

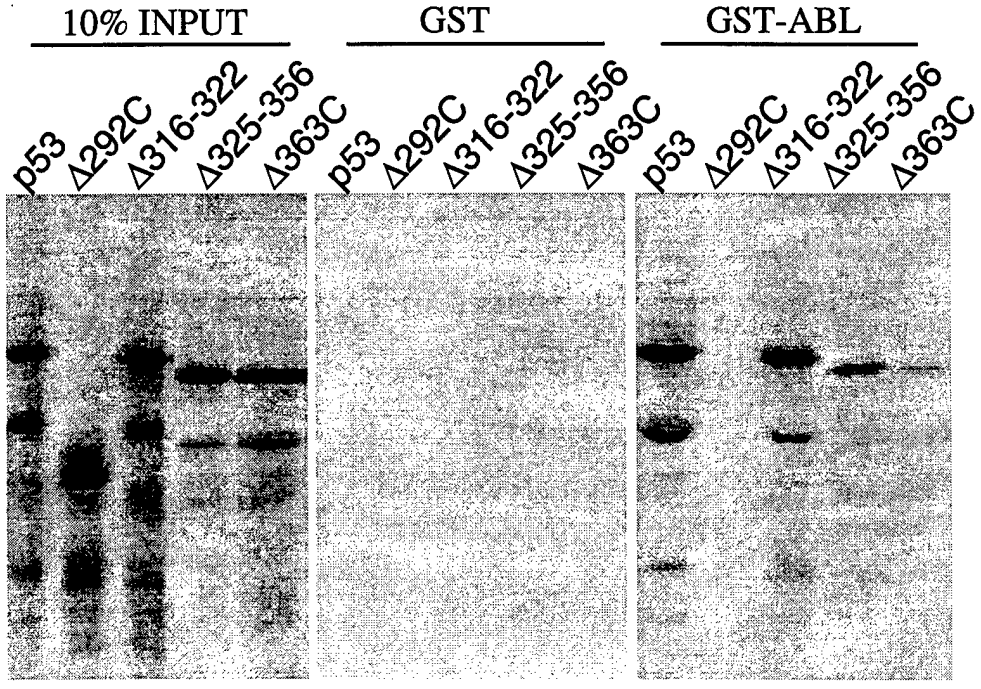

10% INPUT
GST
GST-ABL
p53
Δ292C
Δ316-322
Δ325-356
Δ363C
p53
Δ292C
Δ316-322
Δ325-356
Δ363C
p53
Δ292C
Δ316-322
Δ325-356
Δ363C

FIGURE 1

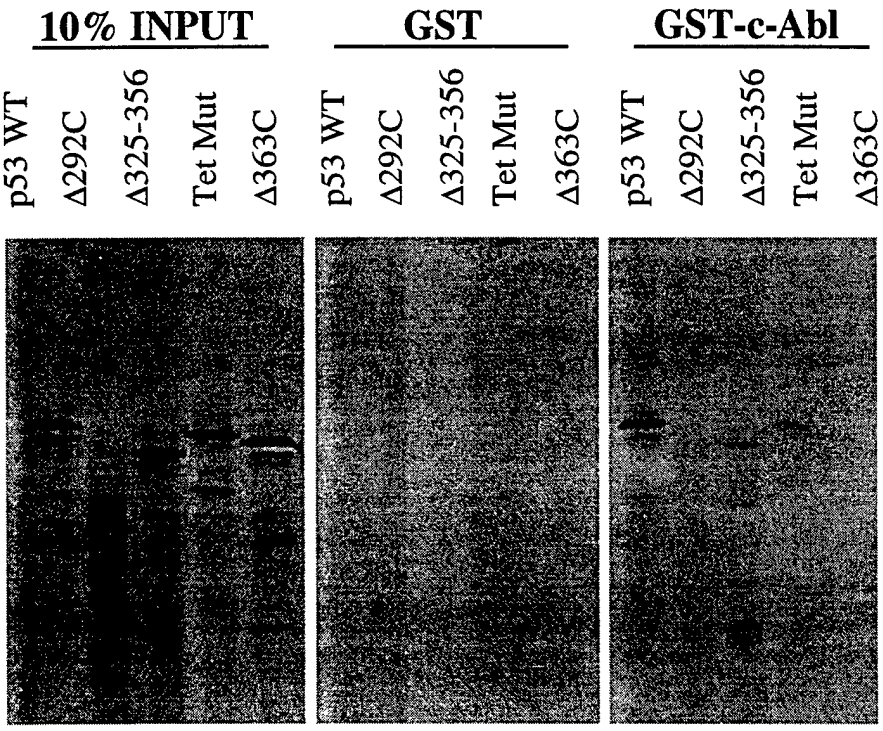

10% INPUT
GST
GST-c-Abl
p53 WT
Δ292C
Δ325-356
Tet Mut
Δ363C
p53 WT
Δ292C
Δ325-356
Tet Mut
Δ363C
p53 WT
Δ292C
Δ325-356
Tet Mut
Δ363C

FIGURE 2

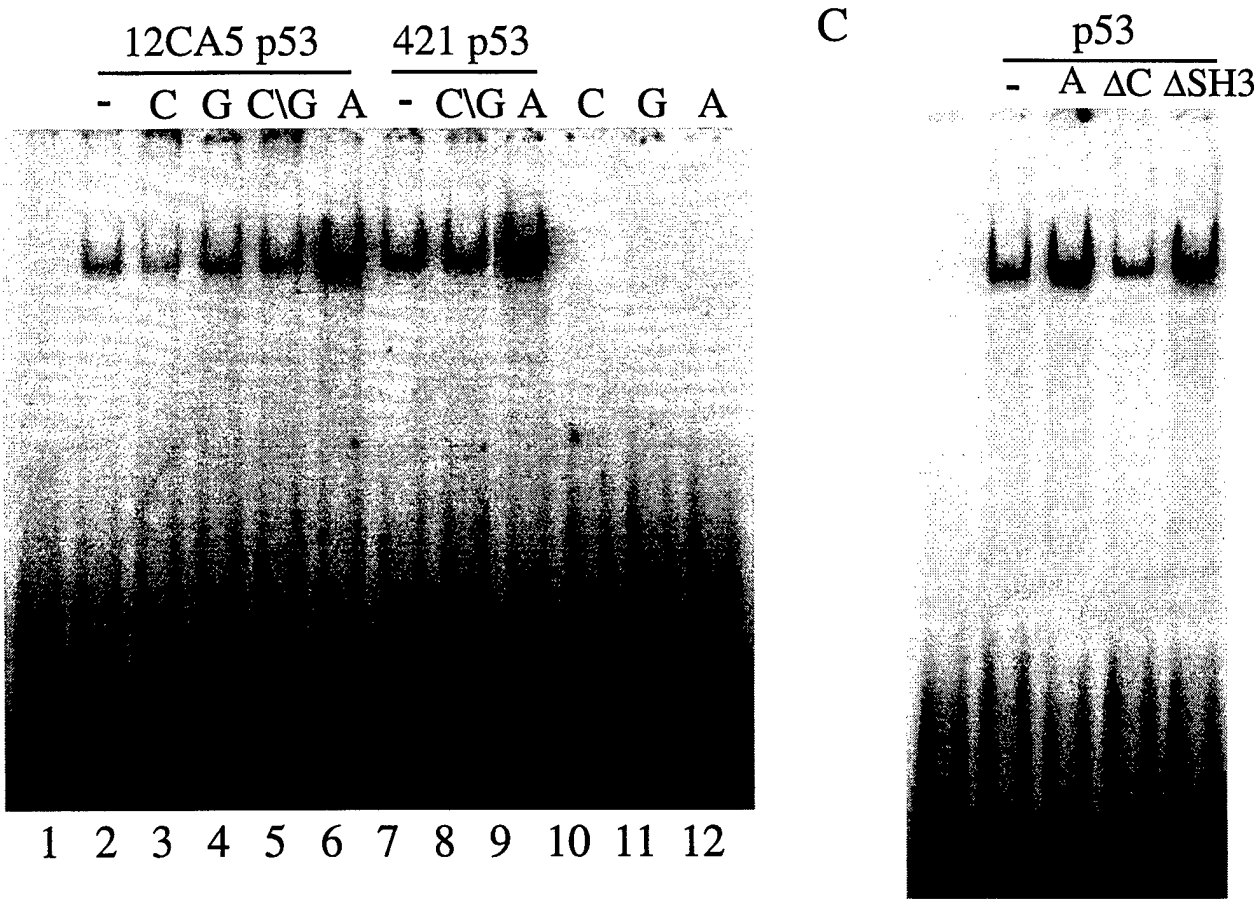

A
12CA5 p53
421 p53
- C G C\G A - C\G A C G A
1 2 3 4 5 6 7 8 9 10 11 12
C
p53
- A ΔC ΔSH3
B
Δ363C
p53
c-Abl
BSA
BSA
200
97
68
43
29
1 2 3 4 5
D
p53
Δ363C
C A C A 2C 2A
1 2 3 4 5 6 7

FIGURE 3

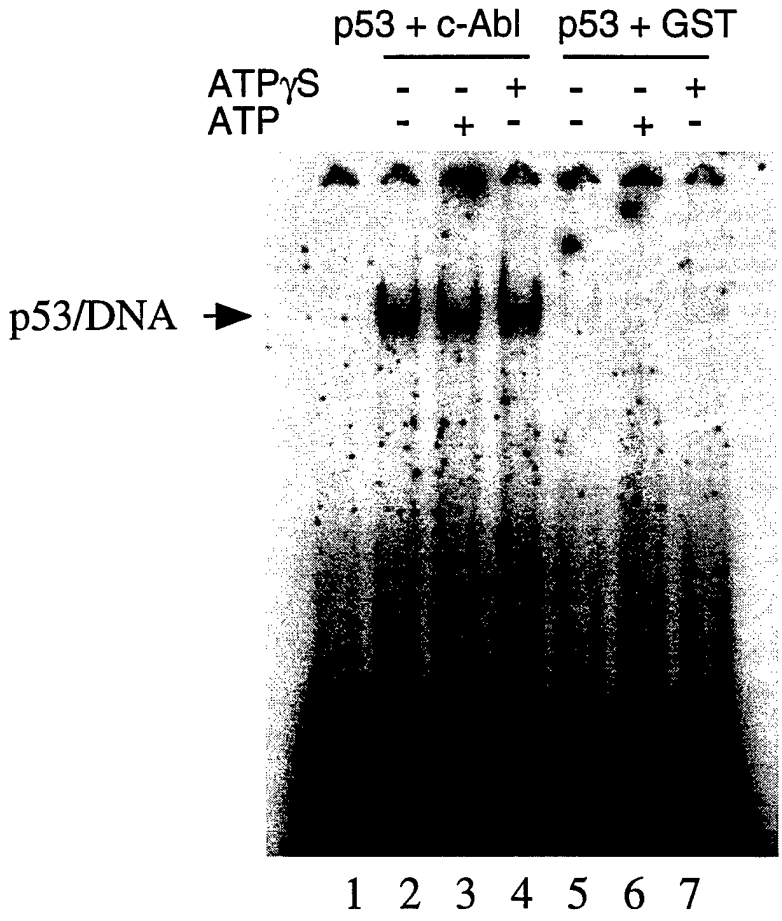
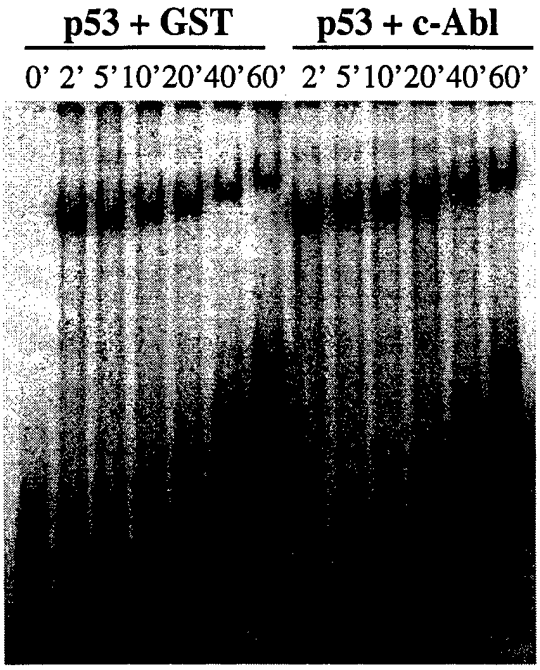


FIGURE 4

A.


p53 + GST
p53 + c-Abl
0' 2' 5' 10' 20' 40' 60' 2' 5' 10' 20' 40' 60'

B.

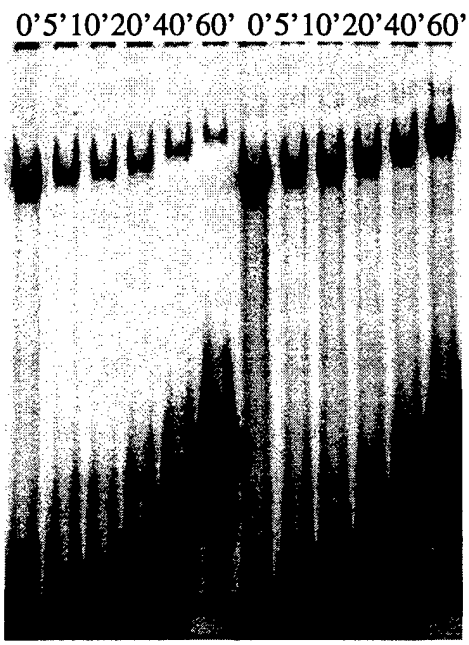
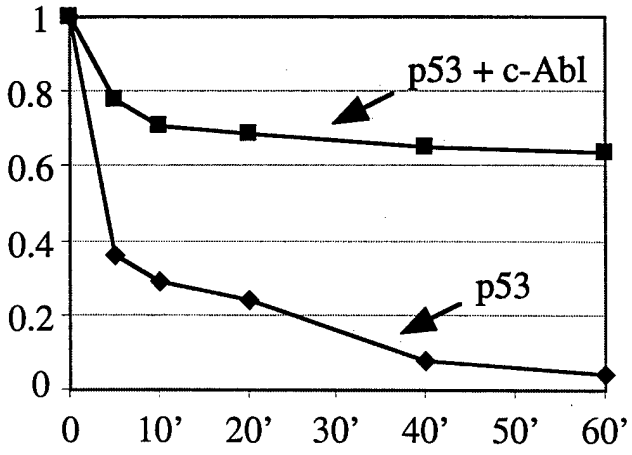

p53+GST+Oligo
p53+c-Abl+Oligo
0' 5' 10' 20' 40' 60' 0' 5' 10' 20' 40' 60'

FIGURE 5

C.


1
0.8
0.6
0.4
0.2
0
0 10' 20' 30' 40' 50' 60'
p53 + c-Abl
p53

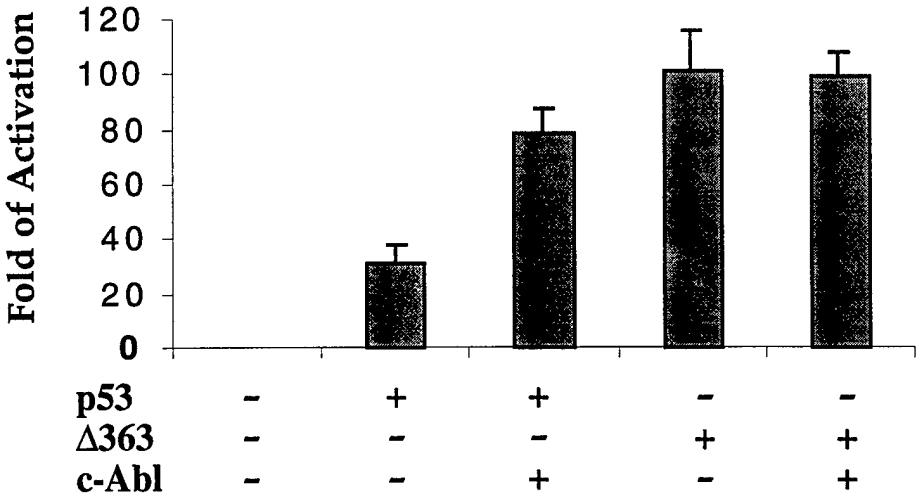


FIGURE 6

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 3, Issue of January 15, pp. 1677–1682, 1999
Printed in U.S.A.
© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

Mitogen-activated Protein Kinase Is Involved in the Degradation of p53 Protein in the Bryostatin-1-induced Differentiation of the Acute Promyelocytic Leukemia NB4 Cell Line*

(Received for publication, September 16, 1998, and in revised form, October 20, 1998)

XinDe Song, Hilary M. Sheppard, Anthony W. Norman, and Xuan Liu‡

From the Department of Biochemistry, University of California, Riverside, California 92521

Overexpression of mutant p53 has been reported to promote tumorigenicity in several cancers. However, despite its potential importance, the signals regulating mutant p53 protein expression are not known. Here we show that a form of p53 that is incapable of binding DNA is overexpressed in the acute promyelocytic leukemia NB4 cell line. Our results demonstrate that treatment of NB4 cells with bryostatin-1, which induces differentiation in this cell line, leads to hyperphosphorylation of this DNA binding-impaired form of p53 via mitogen-activated protein kinase. After this phosphorylation, the p53 protein is degraded by the ubiquitin/proteasome pathway. Furthermore, we show that inhibition of p53 hyperphosphorylation blocks p53 protein degradation and cell differentiation. In addition, inhibition of the ubiquitin/proteasome pathway also blocks p53 protein degradation and cell differentiation. These findings suggest a role for mitogen-activated protein kinase in the degradation of the DNA binding-impaired form of p53 protein and in the bryostatin-induced differentiation observed in this cell line. The implications of these results with respect to the functional significance of p53 phosphorylation and degradation in cell differentiation are discussed.

Studies of human and mouse p53 have shown that wild-type p53 exerts its antiproliferation function by inducing growth arrest and apoptosis, whereas mutant p53 loses this function (1, 2). The biochemical activity of p53 that is required for this relies on its ability to bind to specific DNA sequences and to function as a transcription factor (3). The importance of the activation of transcription by p53 is underscored by the fact that the majority of p53 mutations found in tumors are located within the domain required for sequence-specific DNA binding (1, 2). Therefore, it is clear that this activity is critical to the role of p53 in preventing proliferation. Although the precise molecular mechanisms by which mutant p53 loses its antiproliferation functions remain to be elucidated, three models have been proposed (1). First, mutations in p53 may result in a loss of tumor suppressor function. Second, mutant p53 may have a dominant negative effect over wild-type p53 activity. Finally, mutations in p53 may lead to "a gain of function" such that it can induce proliferation and promote tumorigenicity of various cells (4–6).

An important mechanism used to control p53 activity is the regulation of p53 protein levels. Regulation is primarily achieved via protein degradation, although p53 levels may also be controlled at the level of transcription (7) and translation (8). Studies with human papilloma virus E6 protein and cellular oncogene Mdm2, which interact with and lead to the degradation of p53, have revealed that p53 is degraded by the ubiquitin-mediated proteolytic pathway (9–11). Although signals that target p53 for degradation are not yet fully understood, it is generally accepted that the phosphorylation status of p53 may be involved. As an illustration of this, cells treated with the serine phosphatase inhibitor okadaic acid accumulate high levels of hyperphosphorylated wild-type p53 (12). Clearly, the regulation of p53 protein level is important to its tumor suppressor function. Therefore, it follows that the regulation of mutant p53 protein levels may be important in regulating the oncogenic potential of mutant p53.

In this study we examined the involvement of the p53 pathway in NB4 cell differentiation. The NB4 cell line was originally isolated from a patient with acute promyelocytic leukemia and is characterized by a translocation involving chromosomes 15 and 17 (13). It has been used as a model for studying the mechanisms of cell differentiation, as it can be terminally differentiated into either mature neutrophilic granulocytes (14) or monocyte/macrophage-like cells (15) in response to various treatments. Among the treatments that have been shown to induce NB4 cells to differentiate into monocytes/macrophage-like cells are 1α,25-dihydroxyvitamin D_3 (16) and bryostatin-1 (17). Although the precise mechanisms involved in this differentiation are not yet fully understood, it is clear that a mitogen-activated protein kinase (MAPK)[1] pathway is involved (17, 18). Interestingly, MAPK has been shown to phosphorylate the amino terminus of p53 *in vitro* (19). Thus, a possible mechanism for bryostatin-induced differentiation may involve the phosphorylation of p53 via the MAPK pathway, resulting in an alteration of p53 antiproliferation activity.

To address the question of whether the p53 pathway is involved in bryostatin-induced differentiation, we asked whether endogenous p53 is phosphorylated in response to bryostatin treatment. Our results show that p53 becomes hyperphosphorylated and that the p53 protein is degraded via the ubiquitin/proteasome pathway after treatment with bryostatin-1. Furthermore, we demonstrate that inhibition of p53 hyperphosphorylation by a MAPK pathway inhibitor, PD98059, blocks p53 protein degradation and cell differentiation. In addition, inhibition of p53 protein degradation also blocks cell differentiation. The correlation between these effects suggests a role for MAPK in p53 degradation and in the bryostatin-

* This work was supported by National Institutes of Health Grants CA75180-02 (to X. L.) and DK09012-034 (to A. W. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 909-787-4350; Fax: 909-787-4434; E-mail: xuan.liu@ucr.edu.

[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; EMSA, electrophoresis mobility shift analysis; PAGE, polyacrylamide gel electrophoresis.

induced differentiation in this cell line. To assess the physiological significance of these observations, we examined the DNA binding ability of p53 purified from NB4 cells using anti-p53 antibody, Pab 421. The purified p53 was incapable of binding DNA in electrophoresis mobility shift analysis (EMSA), indicating that a mutant form of p53 exists in NB4 cells. These results suggest that following bryostatin treatment of NB4 cells, the mutant form of p53 is phosphorylated via the MAPK pathway and subsequently degraded. The implication of these observations with respect to the functional significance of p53 phosphorylation and degradation in cell differentiation are discussed.

EXPERIMENTAL PROCEDURES

Cell Culture and Protein Purification—NB4 cells were cultured in Dulbecco's modified Eagle's medium/F-12 media supplemented with 10% fetal bovine serum. The cells were routinely grown as suspension cultures, and passages 8 to 20 were used for each assay. Bryostatin-1 (Alexis, CA) was dissolved in ethanol. PD98095 (Calbiochem) and MG132 (Calbiochem) were dissolved in Me_2SO.

p53 was immunopurified from nuclear extracts prepared from NB4 cells according to the method of Dignam *et al.* (20). One milliliter of nuclear extract (7 mg of protein/ml) was incubated for 3 h at 4 °C with gentle rotation with 100 μl of packed protein A-Sepharose beads covalently coupled with anti-p53 antibody Pab 421. Beads were washed twice with 0.5 M KCl D buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and once with 0.1 M KCl D buffer. p53 was eluted from the washed beads with 100 μl of 421 epitope oligopeptide (KKGQSTSRHKK) at 1 mg/ml concentration in 0.1 M KCl D buffer. Recombinant p53 was prepared from HeLa cells infected with recombinant vaccinia virus expressing p53 (21) as described above. Proteins were analyzed by SDS-PAGE followed by Western analysis or silver-staining to visualize bands.

Detection of p53 Phosphorylation and Protein Level in NB4 Cells—Two ml of NB4 cells at 10^6 cell/ml were labeled with [^{32}P]orthophosphate (ICN) in phosphate-deficient Dulbecco's modified Eagle's medium (Life Technologies, Inc.) and then incubated for 1 h followed immediately by bryostatin-1 treatment. At the end of the treatment, the cells were washed with PBS containing 100 μM vanadate and lysed by adding 0.5 ml of radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 mM Na_3VO_4, 2 mM EGTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.25% sodium deoxycholate, 1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin). The lysate was homogenized by a Dounce homogenizer and clarified by centrifugation at 14,000 × *g* for 15 min at 4 °C. The supernatant were then incubated overnight at 4 °C with 20 μl of packed protein A-Sepharose beads to which Pab 421, an antibody specific for p53, was covalently linked. The immunoprecipitate was subjected to SDS-PAGE, and the labeled proteins were visualized with a PhosphorImager using Adobe Photoshop software. To determine p53 protein levels, NB4 cells were lysed with radioimmune precipitation buffer as described above, and the protein concentration for each sample was measured. Equivalent amounts of cell lysate were analyzed by SDS-PAGE followed by Western blot analysis using Pab 421.

Detection of Activated MAPK—Phosphorylated MAPK was detected by immunoprecipitation with anti-phosphotyrosine monoclonal antibody followed by immunoblotting using anti-MAP kinase antibody as described (18). Briefly, 2 × 10^6 NB4 cells were lysed with 0.5 ml of radioimmune precipitation buffer, and protein concentration was determined with a protein assay kit (Bio-Rad). Supernatant containing an equivalent amount of protein from each sample was incubated with 20 μl of packed agarose beads coupled to a monoclonal anti-phosphotyrosine antibody (PT-66, Sigma) at 4 °C overnight. The immunoprecipitate was then analyzed by SDS-PAGE followed by Western analysis with a rabbit anti-p42 MAPK polyclonal antibody (Pab C14, Santa Cruz). Equal loading of MAP kinase protein was determined by Western blot analysis using anti-p42 MAPK antibody.

EMSA—An oligonucleotide probe containing the ribosomal gene cluster (RGC) p53-binding site was used containing the sequence 5′-AGCTTGCCTCGAGCTTGCCTGGACTTGCCTGGTCGACGC-3′. Binding reactions contained 60 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 10 μg bovine serum albumin, 0.2 μg of poly(d(G-C)), 600 cpm of ^{32}P-labeled probe and proteins as indicated, and water in a total volume of 12.5 μl. Reactions were incubated for 30 min at 30 °C and then analyzed on a 3% polyacrylamide gel containing 0.5 × TBE (0.045 mM Tris borate, 0.045 mM sodium borate, 0.001 mM EDTA, pH 8.0). The gel was dried, and DNA-protein complexes were visualized with a PhosphorImager using Adobe Photoshop software.

TABLE I
Effect of PD98059 and MG132 on bryostatin-induced cell differentiation

Treatments	Adherence	Phagocytosis
	%	%
Control	0.2	0
Bryostatin-1	35	21
PD98059	0.2	0
Bryostatin + PD	3	2
MG132	0.2	0
Bryostatin + MG	0.2	0

Northern Blot Analysis—Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Thirty μg of total RNA was subjected to electrophoresis in a 1.5% agarose gel and transferred to a MAGNA nylon transfer membrane (Micron Separations Inc.). A 1.3-kilobase pair DNA fragment corresponding to the full-length human p53 gene was cut from plasmid pcDNA-p53 (22) and labeled using T7 QuickPrime Kit (Amersham Pharmacia Biotech) as a probe. Northern analysis was conducted using a standard procedure (23). The RNA bands were visualized with a PhosphorImager using Adobe Photoshop software. The membrane was then stripped and hybridized with a glyceraldehyde 3-phosphate dehydrogenase cDNA probe to normalize for RNA loading.

Studies of Cell Differentiation—To examine the effect of bryostatin-1 on cell adherence, 2 × 10^4 cells were seeded in a 24-well plate. Cells were pretreated with Me_2SO control or 5 μM PD98059 or 1 μM MG132 for 30 min and then treated with vehicle or 5.6 nM bryostatin-1 for 72 h. At the end of the treatment, cells in suspension *versus* those adhered to the culture plate were counted. A total of 1000 cells were counted for each treatment, and adherence was expressed as a percentage of adherent cells to the total number of cells. Phagocytosis was measured by incubation of NB4 cells with latex beads (3 μm, Sigma) for 5 h after 72 h treatment of cells with the reagents as described above. Cells were gently washed 4 times and centrifuged at 125 × *g* for 5 min to remove the free beads. A total of 500 cells were counted for each treatment, and phagocytosis was expressed as the percentage of bead-engulfing cells to total cells. Both bead-engulfing cells and total cells were counted under a microscope and photographed.

RESULTS

p53 Hyperphosphorylation and Reduction in NB4 Cell Differentiation—To determine whether the p53 pathway is involved in NB4 cell differentiation, we first investigated whether the phosphorylation status of p53 was altered in response to bryostatin treatment. NB4 cells have been characterized with respect to differentiation induced by bryostatin-1 (Ref. 17; also see Fig. 5 and Table I). Initial experiments suggested that treatment with 5.6 and 56 nM bryostatin-1 induced differentiation. Therefore, NB4 cells were metabolically labeled with $^{32}P_i$ and immediately treated with 0, 0.56, 5.6, or 56 nM bryostatin-1. From each treatment, lysate containing equivalent amounts of protein was immunoprecipitated with anti-p53 antibody. After electrophoresis, the amount of phosphorylation of p53 was analyzed by autoradiography (Fig. 1*A*). In the absence of bryostatin-1, p53 protein showed basal levels of phosphorylation, which is as expected for a phosphoprotein. The addition of bryostatin-1, however, resulted in an increase in phosphorylation above this basal level. The maximum level of phosphorylation was observed after treatment with 5.6 and 56 nM bryostatin-1 (Fig. 1*A*). It was also demonstrated that the maximum level of phosphorylation was obtained 30 min after bryostatin treatment (Fig. 1*B*). A control Western analysis was performed to ensure the equivalent amounts of p53 protein present in each sample (data not shown). Consequently, these results indicate that p53 becomes hyperphosphorylated after bryostatin treatment in a dose- and time-dependent manner.

Next we asked whether bryostatin-1 would affect p53 protein levels, as it has been previously suggested that the phospho-

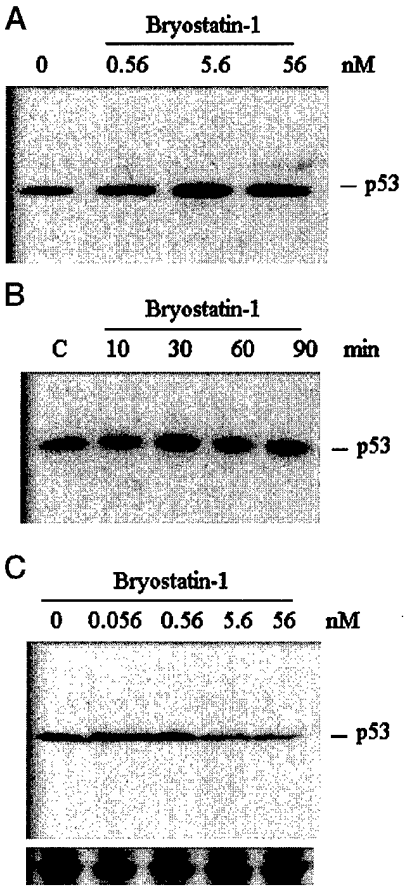


FIG. 1. **Treatment with bryostatin-1 induces p53 protein phosphorylation and reduction in NB4 cells.** NB4 cells were labeled with [^{32}P]orthophosphate and were treated with control or with bryostatin-1 at various concentrations as indicated for 30 min (*A*) or treated with 5.6 nM bryostatin-1 for various time periods as indicated (*B*). Cells extracts were then prepared, and p53 protein was immunoprecipitated using p53 antibody. Protein samples were resolved by electrophoresis on a SDS-PAGE gel and visualized by autoradiography. *C*, to study p53 protein reduction NB4, cells were treated with various concentrations of bryostatin-1 as indicated for 12 h. Cells extracts were then prepared, and Western blot analysis was performed with anti-p53 antibody (*top panel*). Equal loading of protein from crude extract was verified by Coomassie Blue staining (*bottom panel*).

rylation status of p53 may be involved in its targeting for degradation. The NB4 cells were treated with increasing amounts of bryostatin-1 or control vehicle, and the resulting p53 protein level was analyzed by Western analysis (Fig. 1*C*). It was anticipated that the hyperphosphorylation of p53 would result in an increase in p53 protein levels as phosphatase inhibitor treatment results in an accumulation of high levels of wild-type p53 (12). Surprisingly, however, treatment of cells with 5.6 and 56 nM brystatin-1 significantly reduced p53 protein levels. Treatment with 0.056 and 0.56 nM bryostatin-1 had little effect. Coomassie Blue staining of major representative bands indicated that equivalent amounts of protein were present (Fig. 1*C*, *lower panel*). These results suggest that treatment with bryostatin-1 decreases p53 protein levels in NB4 cells. Furthermore, the results indicate that the concentrations of bryostatin-1 sufficient to increase p53 phosphorylation (5.6 and 56 nM) are the same as those required to decrease p53 protein levels. Similarly, those bryostatin concentrations that had little effect on p53 phosphorylation were also not effective in reducing the protein levels. We interpret this correlation to indicate that hyperphosphorylation of p53 might be involved in the reduction of p53 protein levels. It is particularly interesting to note that treatment of NB4 cells with bryostatin-1 at concentrations of 5.6 and 56 nM but not at 0.56 nM has been shown to induce differentiation (Fig. 5 and Table I).[2]

MAPK Pathway Is Involved in p53 Hyperphosphorylation and Reduction in p53 Protein Levels—We have shown that MAPK is activated in NB4 cells after bryostatin treatment,[2] which raised the possibility that the MAPK pathway might be involved in p53 hyperphosphorylation and protein reduction. To address this question, a specific MAPK pathway inhibitor, PD98059 (24), was employed. Phosphorylation of MAPK can be blocked in a dose-dependent manner by this inhibitor, but it is maximally effective at a concentration of 5 μM. Five min after treating NB4 cells with 5.6 nM bryostatin-1, phosphorylation of MAPK (p44 and p42) was significantly increased in the absence of inhibitor (Fig. 2*A*). When 5 μM PD98059 was included with the bryostatin-1 treatment, no detectable increase in MAPK phosphorylation was found. This result confirms that PD98059 can block bryostatin-induced MAPK activation in this cell line. We next examined whether PD98059 was able to block p53 hyperphosphorylation and protein reduction. Fig. 2*B* shows that treatment with 5.6 nM bryostatin-1 increased phosphorylation of p53 and that concurrent treatment with PD98059 blocked this bryostatin-induced p53 hyperphosphorylation in a dose-dependent manner. This is consistent with the hypothesis that MAPK is involved in the bryostatin-induced phosphorylation of p53. Importantly, treatment with PD98059 also inhibited bryostatin-induced p53 protein reduction (Fig. 2*C*). Coomassie Blue staining of major representative bands indicated that equivalent amounts of protein were present (Fig. 2*C*, *lower panel*). Consequently, our data demonstrate that the MAPK pathway is involved in p53 hyperphosphorylation and in the reduction in p53 protein levels. Although direct phosphorylation of p53 by the MAPK pathway remains to be elucidated, it is clear that MAPK is involved. In addition, these results suggest that p53 hyperphosphorylation might be associated with a reduction in p53 protein levels.

Reduction in p53 Protein Levels Is Mediated by Ubiquitin/Proteasome Pathway—We next asked if the reduction in p53 protein levels was because of protein degradation. To address this question, we examined the steady state level of p53 mRNA by Northern analysis. NB4 cells were treated with either 5.6 nM brystatin-1 or control vehicle, and mRNA was extracted at various time points as indicated (Fig. 3, *top*). As a control to ensure equal loading of RNA, a probe specific for glyceraldehyde 3-phosphate dehydrogenase was used (Fig. 3, *bottom*). Our results demonstrate that the reduction in p53 protein levels was not caused by a decrease in p53 mRNA levels. This implied that the reduction in p53 protein levels might be caused by a decrease in protein stability.

To test this hypothesis, sodium borohydride ($NaBH_4$), an inhibitor of ubiquitin COOH-terminal hydrolase, was used to determine whether an inhibition of the ubiquitin/proteasome pathway would block the bryostatin-induced decrease in p53 protein levels. Ubiquitin COOH-terminal hydrolase is required for the generation of the functional monomeric form of ubiquitin (25) and is suggested to play a role in bryostatin-induced Reh cell differentiation (26). Our results show that bryostatin-induced p53 reduction was completely inhibited by sodium borohydride (Fig. 3*B*). As proteasomes play a key role in protein degradation, we then tested whether a proteasome inhibitor, MG132, could inhibit p53 degradation. Fig. 3*B* shows that, like sodium borohydride, MG132 also blocks bryostatin-induced p53 reduction. Taken together, these results suggest that the MAPK-mediated decrease in p53 protein levels is not caused by decreased gene transcription but by ubiquitin/proteasome-de-

[2] X-D. Song and A. W. Norman, unpublished data.

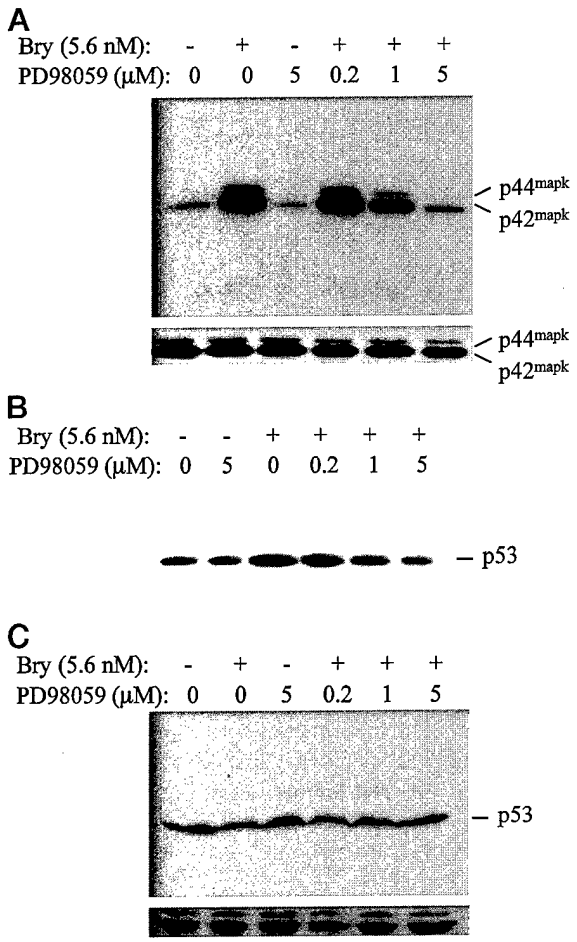


FIG. 2. **MAP kinase is involved in p53 phosphorylation and degradation in NB4 cells.** *A*, after a 30-min preincubation in the presence or absence of MAPK pathway inhibitor PD98059, NB4 cells were treated with control or 5.6 nM bryostatin-1 (*Bry*) for 5 min. Cells extracts were then prepared, tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibody, and protein samples were resolved by electrophoresis on a SDS-PAGE gel. Western blotting was then performed with anti-MAP kinase antibody (*top panel*). To determine total levels of MAPK protein present in the cells, Western blotting with anti-MAP kinase antibody was performed on cell extracts (*bottom panel*). *B*, NB4 cells were labeled with [^{32}P]orthophosphate, PD98059 was added at various concentrations as indicated, either in the presence or absence of 5.6 nM bryostatin-1, and incubation was continued for a further 30 min. Cells extracts were then prepared, and p53 protein was immunoprecipitated. Protein samples were resolved by electrophoresis on a SDS-PAGE gel and visualized by autoradiography. *C*, NB4 cells were preincubated with PD98059 at various concentrations as indicated, after which control or 5.6 nM bryostatin-1 was added, and incubation was continued for 12 h. Cells extracts were then prepared, and Western blotting was performed with anti-p53 antibody. Equal loading of protein from crude extract was verified by Coomassie Blue staining (*bottom panel*).

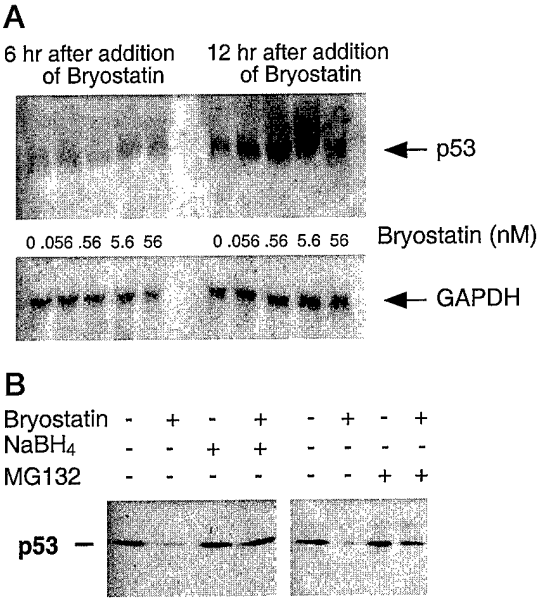


FIG. 3. **The reduction in p53 protein levels is because of protein degradation.** *A*, NB4 cells were treated with control vehicle or with bryostatin-1 at various concentrations as indicated for 6 or 12 h, after which total RNA was extracted. Thirty μg of total RNA from each sample were subjected to Northern blot analysis using a probe specific for p53 mRNA (*upper panel*). To ensure equal loading of RNA, the membrane was stripped and re-incubated with a probe specific for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA *B*, to study the effect of inhibitors of the ubiquitin/proteosome pathway on bryostatin-1-induced p53 degradation, NB4 cells were preincubated with sodium borohydride ($NaBH_4$) or MG132 for 30 min, after which 5.6 nM bryostatin-1 was added, and incubation was continued for 12 h. Cells extracts were then prepared, and Western blotting was performed with anti-p53 antibody.

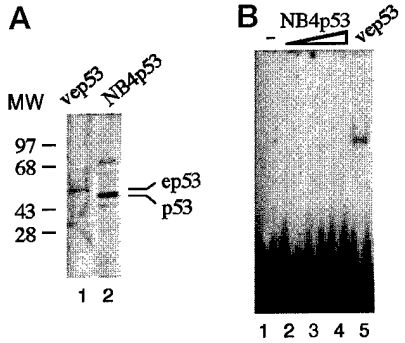


FIG. 4. **p53 purified from NB4 cells fails to bind to DNA in EMSA.** *A*, vaccinia virus expressed human p53 (vep53) purified from HeLa cell extracts and endogenous p53 purified from NB4 cell extracts were subjected to electrophoresis on a SDS-PAGE gel and visualized by silver staining. *B*, in EMSA, radiolabeled probe containing the p53 site from the ribosomal gene cluster was incubated with approximately 50, 75, or 100 ng of p53 purified from NB4 cells (*lanes 2–4*) or 20 ng of vep53 (*lane 5*) before electrophoresis on a 3% polyacrylamide gel. The position of vep53 and p53 expressed from the endogenous genes are shown. *MW*, molecular mass.

pendent protein degradation.

p53 from NB4 Cells Is Incapable of Binding DNA—In an effort to assess the functional significance of MAPK-mediated p53 phosphorylation and degradation in NB4 cell differentiation, we tested the ability of p53 purified from NB4 cells to bind DNA using EMSA. p53 was purified from nuclear extracts prepared from NB4 cells using anti-p53 antibody, Pab 421 (Fig. 4*A*, *lane 2*). This antibody recognizes an epitope in the carboxyl terminus of p53 and is thought to convert p53 from its latent to its active state and thereby significantly increase its DNA binding activity (27). Vaccinia virus expressed epitope-tagged human p53 purified from HeLa cells using the same antibody was used as a control (vep53, Fig. 4*A*, *lane 1*). When a DNA probe containing the p53 cis element identified in the ribosomal gene cluster was incubated with vep53, a shifted band was observed (Fig. 4*B*, *lane 5*). This band was supershifted by the addition of anti-p53 antibody N-19 (Santa Cruz), and the addition of a 100-fold excess of cold ribosomal gene cluster DNA fragment was sufficient to inhibit its formation, suggesting that this band was p53-specific (data not shown). In comparison to wild-type p53, p53 purified from NB4 cells had a significantly reduced affinity for DNA binding. The inability of this p53 to bind DNA suggests that either mutations exist within the protein or that the protein has been inactivated by post-

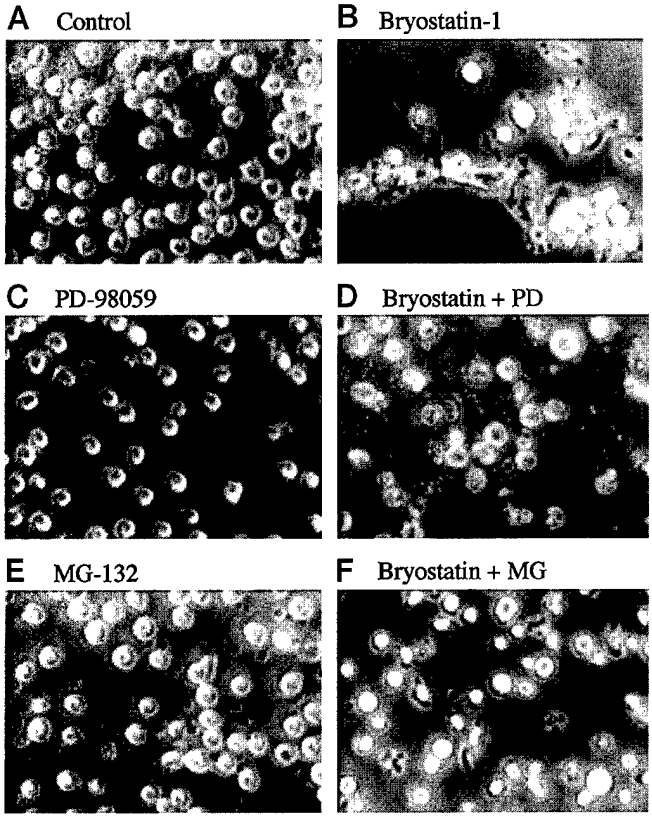


FIG. 5. **MAPK-mediated p53 degradation is associated with cell differentiation.** NB4 cells were treated with control (*A*), 5.6 nM bryostatin-1 (*B*), 5 μM PD98059 (*C*), bryostatin-1 and PD98059 (*D*), 1 μM MG132 (*E*), bryostatin-1 and MG132 (*F*) for 72 h. After each treatment, phagocytosis and cell adherence, the markers for NB4 cell differentiation, were measured by incubation of NB4 cells with latex beads for 5 h, and the cells were photographed with 400× magnification using a Nikon microscope.

translational modification. Regardless of the nature of the alteration, these results establish that p53 in NB4 cells is unable to bind DNA. It may be, therefore, that degradation of this DNA binding-impaired form of p53 is associated with NB4 cell differentiation.

MAPK-mediated p53 Degradation Is Associated with Bryostatin-induced NB4 Cell Differentiation—To demonstrate the physiological significance of the p53 degradation in NB4 cell differentiation, we tested whether the proteasome inhibitor, MG132, would block bryostatin-induced differentiation. Untreated NB4 cells grow as a suspension and contain nonadherent cells (Fig. 5*A*). When treated with 5.6 nM bryostatin-1, 35% of the cells become attached to the surface of the culture plate (Table I), and 21% exhibit a morphology related to monocyte/macrophages (Fig. 5*B*), which is the marker for NB4 cell differentiation. In contrast, concurrent treatment with MG132 blocked both the bryostatin-induced cell adherence (Table I) and phagocytotic activities (Fig. 5*F*), suggesting that inhibition of the ubiquitin/proteasome pathway blocked NB4 cell differentiation. Treatment with MG132 alone had little effect on NB4 cell differentiation (Fig. 5*E* and Table I) or cell viability (data not shown).

The observation that the MAPK pathway is involved in phosphorylation/degradation of a mutant form of p53 suggested that the MAPK pathway might be involved in cell differentiation. Therefore, we tested whether the MAPK pathway inhibitor, PD98059, could inhibit bryostatin-induced NB4 cell differentiation under the same conditions that were sufficient to induce p53 phosphorylation/degradation. Concurrent treatment of NB4 cells with bryostatin-1 and 5 μM PD98059 blocked the bryostatin-induced cell adherence (Table I) and phagocytotic activities (Fig. 5*D*), suggesting that inhibition of MAPK blocked NB4 cell differentiation. Treatment with 5 μM PD98059 alone had no effect on NB4 cell differentiation (Fig. 5*C* and Table I) or cell viability (data not shown). This observation supports the view that the MAPK pathway is involved in bryostatin-induced NB4 cell differentiation. The critical requirement for MAPK in differentiation coupled with the demonstration that MAPK is involved in phosphorylation/protein degradation of a mutant p53 suggests that degradation of mutant p53 may possibly play a role in cell differentiation.

DISCUSSION

A requisite first step toward understanding the molecular mechanism for cell differentiation and proliferation is to identify the signal transduction pathways and the cellular targets of those pathways involved in these processes. In this paper we present biochemical evidence that indicates that the MAPK pathway, required for bryostatin-induced cell differentiation, induces p53 hyperphosphorylation, which subsequently reduces p53 protein stability. This observation is particularly significant because an altered form of p53, unable to bind DNA *in vitro*, is overexpressed in NB4 cells. It has been suggested that mutant p53 may gain a function such that it can promote tumorigenicity in various cells. Consequently, degradation of mutant p53 protein may result in a reduction of tumorigenicity. Taken together, our results indicate that an altered form of p53 is hyperphosphorylated via the MAPK pathway and subsequently degraded. Although a direct cause and effect relationship is yet to be established, our findings indicate that a reduction in mutant p53 levels may contribute to cell differentiation.

Phosphorylation is one potential mechanism by which cells might regulate p53 protein levels and, hence, its antiproliferation activity. Experiments *in vivo* have clearly demonstrated that p53 is a phosphoprotein on which multiple phosphorylation sites have been identified (1). In comparison to wild-type p53, literature relating to the phosphorylation of mutant p53 and the control of its protein level is limited. In this study we provide *in vivo* evidence that a form of p53 which is incapable of binding DNA is hyperphosphorylated and subsequently degraded via the MAPK pathway, although it is not clear from these experiments whether MAPK phosphorylates this DNA binding-impaired form of p53 directly or whether additional kinases are required. Furthermore, our results suggest that phosphorylation of p53 leads to a reduction in protein stability mediated by the ubiquitin/proteasome pathway.

The observation of a reduction in protein stability after phosphorylation is in contrast to previous reports in which phosphorylation of p53 led to an increase in its stability (12, 28). However, in agreement with our findings, two proto-oncogene-encoded transcription factors, c-Jun and BCL-6, have also been shown to be degraded after phosphorylation by MAPK (29–30). Thus, degradation of oncogene products, including mutant p53, may represent a general mechanism by which the MAPK pathway controls cell function. Reduction of p53 protein levels was observed 12 h after bryostatin treatment, which is slower than the 2 h reported for BCL and c-Jun. It is possible that MAPK may mediate a second signal required for the reduction of p53 protein levels. Nevertheless, our data show that a strong correlation exists between p53 protein phosphorylation and stability.

The finding that mutant p53 is phosphorylated via the MAPK pathway and subsequently degraded has several implications when considering the model in which mutations in p53 lead to a gain of function that promotes tumorigenicity. First, overexpression of mutant p53 in pre-B (31), fibroblast (5), and osteosarcoma cells (32) has been shown to dramatically en-

hance the tumorigenicity of these cells. Reduction in p53 protein levels, therefore, may be of significance in preventing cell proliferation. Indeed, our data demonstrate a strong correlation between protein degradation and cell differentiation. Second, the high frequency of p53 mutations in human cancers warrants a detailed analysis of the molecular mechanisms of the gain of function of mutant p53 and the signals that may regulate this function. In this paper we go some way toward this goal by presenting evidence that the MAPK pathway is involved in degradation of the DNA binding-impaired form of p53 and by correlating this function with NB4 cell differentiation. Finally, a significant research effort is concerned with finding strategies to inactivate mutant p53. In light of the results presented here, it may also be effective to develop therapies designed to reduce mutant p53 protein levels.

Acknowledgments—We thank Dr. Francey Sladek for her valuable comments and critical reading of the manuscript.

REFERENCES

1. Ko, L. J., and Prives, C. (1996) *Genes Dev.* **10,** 1054–1072
2. Levine, A. J. (1997) *Cell* **88,** 323–331
3. Pietenpol, J. A., Tokino, T., Thiagalingam, S., Ei-Deiry, W. S., Kinzler, K. W., and Vogelstein, B. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1998–2002
4. Zambetti, G. P., Bargonetti, J., Walker, K., Prives, C., and Levine, A. J. (1992) *Genes Dev.* **6,** 1143–1152
5. Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A. K., Moore, M., Finlay, C., and Levine, A. J. (1994) *Nat. Genet.* **4,** 42–46
6. Frazier, W. M., He, X., Wang, J., Gu, Z., Cleveland, J. L., and Zambetti, G. P. (1998) *Mol. Cell. Biol.* **18,** 3735–3743
7. Sun, X., Shimizu, H., and Yamamoto, K. I. (1995) *Mol. Cell. Biol.* **15,** 4489–4496
8. Mosner, J., Mummenbrauer, J., Bauer, C., Sczakiel, G., Grosse, F., and Deppert, W. (1995) *EMBO* **14,** 4442–4449
9. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* **387,** 296–299
10. Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997) *Nature* **387,** 299–303
11. Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol. D., Mantovani, F., Breuer, J., Leigh, I. M., Matlashewski, G., and Banks, L. (1998) *Nature* **393,** 229–234
12. Zhang, W., McClain, C., Gau, J. P., Guo, X. Y., and Deisseroth, A. B. (1994) *Cancer Res.* **54,** 4448–4453
13. Lanotte, M., Martin-Thouvenin, V., Najman, S., Ballerini, P., Valensi, F., and Berger, R. (1991) *Blood* **77,** 1080–1088
14. Gianni, M., Terao, M., Norio, P., Barbui, T., Rambaldi, A., and Garattini, E. (1995) *Blood* **85,** 3619–3635
15. Bhatia, M., Kirkland, J. B., and Meckling-Gill, K. A. (1994) *Leukemia (Baltimore)* **8,** 1744–1749
16. Song, X.-D., and Norman, A. W. (1998) *Leuk. Res.* **22,** 69–76
17. Song, X., and Norman, A. W. (1999) *Leukemia*, in press
18. Song, X.-D., Bishop, J. E., Okamura, W. H., and Norman, A. W. (1998) *Endocrinology* **139,** 457–46520
19. Milne, D. M., Campbell, D. G., Caudwell, F. B., and Meek, D. W. (1994) *J. Biol. Chem* **269,** 9253–9260
20. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489
21. Liu, X., and Berk, A. J. (1995) *Mol. Cell. Biol.* **15,** 6474–6478
22. Liu, X., Miller, C. M., Koeffler, H. P., and Berk, A. J. (1993) *Mol. Cell. Biol.* **13,** 3291–3300
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Pang, L., Sawada, T., Decker, S. J., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 13585–13588
25. Wilkinson, K. D., Lee, K. M., Deshpande, S., Duerksen-Hughes, P., Boss, J. M., and Pohi, J. (1989) *Science* **246,** 670–673
26. Mohammad, R. M., Maki, A., Pettit, G. R., and Al-Katib, A. M. (1996) *Enzyme Protein* **49,** 262–272
27. Hupp, T. R., Sparks, A., and Lane, D. P. (1995) *Cell* **83,** 237–245
28. Shieh, S.-Y., Ikeda, M., Taya, Y., and Prives, C. (1997) *Cell* **91,** 325–334
29. Musti, A. M., Treier, M., and Bohmann, D. (1997) *Science* **275,** 400–402
30. Niu, H., Ye, B. H., and Dalla-Favera, R. (1998) *Genes Dev.* **12,** 1953–1961
31. Shaulsky, G., Goldfinger, N., and Rotter, V. (1990) *Mol. Cell. Biol.* **10,** 6565–6577
32. Sun, Y., Nakamura, K., Wendel, E., and Colburn, N. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2827–2831

MOLECULAR AND CELLULAR BIOLOGY, Apr. 1999, p. 2746–2753 Vol. 19, No. 4
0270-7306/99/$04.00+0
Copyright © 1999, American Society for Microbiology. All Rights Reserved.

New Insights into the Mechanism of Inhibition of p53 by Simian Virus 40 Large T Antigen

HILARY M. SHEPPARD, SISKA I. CORNEILLIE, CHRISTINE ESPIRITU, ANDREA GATTI, AND XUAN LIU*

Department of Biochemistry, University of California, Riverside, California 92521

Received 2 November 1998/Returned for modification 9 December 1998/Accepted 19 December 1998

Simian virus 40 (SV40) large tumor antigen (T antigen) has been shown to inhibit p53-dependent transcription by preventing p53 from binding to its cognate *cis* element. Data presented in this report provide the first direct functional evidence that T antigen, under certain conditions, may also repress p53-dependent transcription by a mechanism in which the transactivation domain of p53 is abrogated while DNA binding is unaffected. Specifically, p53 purified as a complex with T antigen from mouse cells was found to bind DNA as a transcriptionally inactive intact complex, while that purified from human cells was found to bind DNA independently of T antigen and could activate p53-dependent transcription. This difference in activity may be dependent on a different interaction of T antigen with mouse and human p53 and, in addition, on the presence of super T, which is found only in transformed rodent cells. These results suggest that subtle yet important differences exist between the inhibition of p53 by T antigen in mouse and human cells. The implications of this finding with respect to SV40-associated malignancies are discussed.

p53 is an important tumor suppressor gene, found to be mutated or absent in over 50% of all cancers studied (23). It functions as a sequence-specific DNA-binding transcription factor (21, 24). In response to double-stranded DNA breaks, p53 is converted from a latent to an active form (17). This results in increased expression of p53-responsive proteins such as p21 which are required for growth arrest at the G_1-to-S phase transition (12). It also mediates apoptosis via the increased expression of proteins such as Bax (30). Inactivation of p53, therefore, results in the loss of a cell cycle checkpoint required for repair of damaged DNA and prevents apoptosis in response to severe DNA damage. In the absence of these responses, oncogenic mutations which may result in tumor progression can accumulate. From the above, it is clear that the transcriptional activation function of p53 is critical to its role as a tumor suppressor.

A number of proteins bind to p53 and negatively affect its transcriptional activity. The cellular oncoprotein MDM2 has been shown to inhibit p53 via three different mechanisms. First, when bound to p53, MDM2 conceals the activation domain of p53 from the transcription machinery, thereby indirectly repressing p53-dependent transcription (32). Second, it has been found to promote the rapid degradation of p53 via a ubiquitin-proteosome pathway, resulting in decreased levels of p53 available to activate transcription (15, 22). Finally, MDM2 may itself function as an active repressor of transcription, which, via its interaction with p53, represses p53-responsive genes (39).

Proteins encoded by DNA tumor viruses also inhibit p53 activity in similar ways. The human papillomavirus type 16 E6 protein forms a complex with p53, thus promoting its polyubiquitination and subsequent degradation (35, 38). The adenovirus early 1B (E1B) 55K protein, a transcriptional repressor, binds to p53 and is thereby targeted to p53-responsive genes (41). The large tumor antigen (T antigen) of simian virus 40 (SV40) also forms with p53 a complex that inhibits p53 function in SV40-infected and SV40-transformed cells. Experiments performed with baculovirus-expressed human p53 and T antigen led Bargonetti et al. (1) to propose that T antigen inhibits p53 function by preventing it from binding to its cognate *cis* element. Furthermore, Segawa et al. (36) reported similar results when they examined the DNA-binding activity of p53 in crude nuclear extracts isolated from a human p53 null cell line transiently transfected with plasmids expressing p53 and T antigen; in addition, when baculovirus-expressed T antigen was added to a mouse cell lysate containing wild-type p53, DNA binding was abolished. Taken together, data derived from these experiments support the model in which T antigen inhibits p53 function by preventing it from binding to DNA.

T antigen is often found as a 90-kDa protein in the nuclei of SV40-infected and/or -transformed cells. However, higher-molecular-weight forms of T antigen, designated super T, have also been detected in SV40-transformed rodent cell lines (37). Forms of super T are reported to arise from internal in-phase duplications in the coding region of the T-antigen gene (28, 29) or, as is the case for a commonly occurring 100-kDa form, by differential splicing between two integrated partial copies of the T-antigen gene (25). The duplication which forms the 100-kDa protein includes the first exon, the intron, and part of the second exon upstream of the complete coding sequence for the T-antigen gene. It is proposed that transcription starts at the upstream copy of T antigen and continues through the host DNA and into the full-length copy of the gene. The long primary transcript is spliced, but a short region of extra RNA, possibly from the first copy of the duplicated control region, is retained and encodes the extra amino acids present in the 100-kDa super-T protein (25). The presence of super T was found to correlate with anchorage-independent growth in mouse cell lines (2, 6). Despite the compelling effect of super T in transformation, the molecular mechanism by which super T functions to inhibit p53-dependent transcription remains to be elucidated.

In the present study, we show that p53 immunopurified from a human cell line and a monkey cell line copurifies with T antigen, while that purified from two mouse cell lines copuri-

* Corresponding author. Mailing address: Department of Biochemistry, University of California, Riverside, CA 92521. Phone: (909) 787-4350. Fax: (909) 787-4434. E-mail: xuan.liu@ucr.edu.

fies with a 100-kDa form of super T. When purified from mouse cells, the p53-T antigen complex can bind specifically to DNA in electrophoretic mobility shift analysis (EMSA). However, despite this DNA-binding activity, the complex is not capable of activating p53-dependent transcription in vitro. Therefore, our data suggest that in mouse cell lines, T antigen/super T abrogates the transactivation domain of p53 and does not affect DNA binding. When purified from either a human or a monkey cell line, the p53-T antigen complex also binds specifically to DNA in EMSA, but surprisingly T antigen is not present in the resulting shifted complex and hence this complex can support p53-dependent transcription in vitro. This finding suggests that in human and monkey cells, p53 may not be inhibited by T antigen. The different activities of the p53-T antigen complex purified from mouse cell lines and from human and monkey cell lines were found to be dependent on a different interaction of T antigen with mouse and human p53 and, in addition, possibly on the presence of super T. These results suggest for the first time the existence of subtle differences between the inhibition of p53 by T antigen in human and mouse cells that may have physiologically significant consequences.

MATERIALS AND METHODS

Protein purification. p53-T antigen complex was immunopurified from nuclear extracts prepared by the method of Dignam et al. (11). One milliliter of nuclear extract (7 mg of protein/ml) was incubated for 3 h at 4°C with 100 μl of packed protein A-Sepharose beads to which Pab 421, a monoclonal antibody specific for p53, was covalently linked. Beads were washed twice with 0.5 M KCl D buffer (20 mM HEPES [pH 7.9], 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and once with 0.1 M KCl D buffer. p53 was eluted from the washed beads with 100 μl of 421 epitope oligopeptide (KKGQSTSR HKK) at 1 mg/ml in 0.1 M KCl D buffer. p53-T antigen complex was also purified by using beads to which Pab 108, a monoclonal antibody specific for T antigen, was covalently linked. In this case the complex was eluted with EG (ethylene glycol) buffer (50% EG, 0.5 M NaCl, 10% glycerol, 20 mM Tris HCl [pH 8.5], 1 mM EDTA) and dialyzed overnight against 0.1 M KCl D buffer. To purify p53 in the absence of T antigen, Pab 421 anti-p53 beads were incubated with nuclear extract and washed as described above. T antigen was eluted from the bound p53 by two 10-min washes in 0.1 ml of 2 M urea in 0.1 M KCl D buffer. The T-antigen-containing supernatant was dialyzed overnight against 0.1 M KCl D buffer. The remaining beads were washed overnight with 0.1 M KCl D buffer, after which p53 was eluted as described above. To immunodeplete the Pab 421-purified complex, a volume of covalently linked Pab 108 beads equal to one-fifth of the protein sample volume was added, and incubation was carried out for 3 h at 4°C with gentle rotation. The supernatant was retained and incubated with fresh Pab 108 beads for a further 3 h, after which the supernatant was collected. A control human p53 (no T antigen present) was prepared from HeLa cells infected with recombinant vaccinia virus expressing p53 as described previously (26) and purified with Pab 421 anti-p53 beads as described above. Recombinant T antigen was prepared from *Spodoptera frugiperda* SF21 insect cells infected with recombinant baculovirus (a gift from C. Prives, Columbia University) as described by Bargonetti et al. (1). Proteins were analyzed by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels which were subjected to Western blotting or were silver stained to visualize bands.

EMSA. The sequence of the oligonucleotide probe containing the ribosomal gene cluster (RGC) p53-binding site is 5′-AGCTTGCCTCGAGCTTGCCTGG ACTTGCCTGGTCGACGC-3′; the sequence of that containing the p53-binding site from the p21 promoter is 5′-AGCTTAATTCTCGAGGAACATGTCCCA ACATGTTGCTCGAGG-3′. Probes were labeled with the Klenow fragment of *Escherichia coli* DNA polymerase. When required, preincubation reactions were performed for 20 min at 4°C prior to EMSA. Binding reaction mixtures contained 60 mM KCl, 12% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 μg of bovine serum albumin, 0.5 μg of poly(dG-dC), 200 pg of ^{32}P-labeled probe, proteins and antibodies as indicated, and water in a total volume of 12.5 μl. Antibody N-19 (Santa Cruz Biotechnology Inc.), recognizing an amino-terminal epitope mapping to within residues 2 to 20, was used against p53. Pab 108, recognizing an amino-terminal epitope mapping to within residues 1 to 82, was used against T antigen. Reaction mixtures were incubated for 30 min at 30°C and then analyzed on a 3% polyacrylamide gel containing 0.5× TBE (0.045 mM Tris-borate, 0.045 mM sodium borate, 0.001 mM EDTA [pH 8.0]). Electrophoresis was carried out in 0.5× TBE. The gel was dried, and DNA-protein complexes were visualized with a PhosphorImager using Adobe Photoshop software. Densitometry was performed with ImageQuant software.

In vitro transcription. Reactions were performed as described previously (26). Briefly, 70 μg of HeLa cell nuclear extract (7 mg of protein/ml) and 150 ng of a synthetic target promoter containing five p53-responsive sites immediately upstream of the adenovirus E4 TATA box and chloramphenicol acetyltransferase (CAT) reporter gene (5RGCE4CAT) were mixed in a final volume of 50 μl with 60 mM KCl, 12 mM HEPES (pH 7.9), 12% glycerol, 6 mM $MgCl_2$, 0.4 mM ribonucleoside triphosphates, 7 mM β-mercaptoethanol, p53, and T antigen as indicated and incubated at 30°C for 60 min. Control reactions were performed with 150 ng of synthetic promoter containing five GAL4-binding sites immediately upstream of the adenovirus E4 TATA box fused to CAT (5GAL4E4CAT) (5) in the presence of 100 ng of bacterially expressed GAL4-VP16 protein. Reactions were stopped by the addition of 50 μl of stop buffer (2% SDS, 200 mM NaCl, 20 mM EDTA, 20 μg of tRNA per ml, 100 μg of proteinase K per ml) followed by incubation at 39°C for 10 min. After phenol-chloroform extraction, the RNA was ethanol precipitated. Primer extension was performed by resuspending the RNA pellet in 10 μl of annealing buffer (125 mM KCl, 25 mM Tris-HCl [pH 8.3], 1,000 cpm of radiolabeled primer) and incubating it at 70°C for 10 min and then at 39°C for 30 min. Twenty-four microliters of extension buffer (5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris HCl [pH 8.3], 0.3 mM deoxynucleoside triphosphates, 10 mM dithiothreitol, 10 U of Moloney murine leukemia virus reverse transcriptase) was then added, and the mixture was incubated for 30 min at 39°C. The reaction was stopped by the addition of 35 μl of stop buffer; primer extension products were ethanol precipitated and resuspended in 4 μl of formamide sequencing dye prior to electrophoresis on a 10% acrylamide-urea gel. The gel was dried, and primer extension products were visualized with a PhosphorImager using Adobe Photoshop software. Densitometry was performed with ImageQuant software.

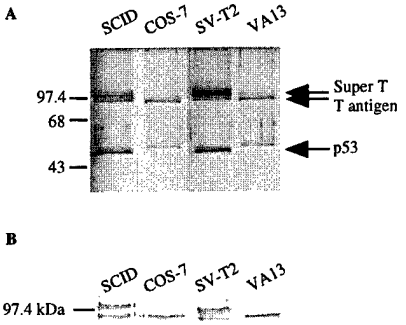


FIG. 1. p53 immunopurified from SV40-transformed cells copurifies with T antigen. (A) Silver-stained SDS-polyacrylamide gel of the p53-T antigen complex purified from four SV40-transformed cell lines derived from mouse (SCID and SV-T2), human (WI38 VA13), and monkey (COS-7) cells. (B) Western blot of the p53-T antigen complex, using anti-T antigen antibody Pab 108, indicating that two forms of T antigen (regular T antigen and super T) are present in the mouse cell lines.

RESULTS

p53-T antigen complex can specifically bind to DNA. To define how T antigen inhibits p53-mediated transcription, p53 was purified from nuclear extracts prepared from two mouse (SCID and SVT2), one monkey (COS-7), and one human (WI38 VA13) cell line, all of which are stably transformed with SV40, using anti-p53 antibody Pab 421 (Fig. 1A). From the mouse cell lines, two proteins with apparent molecular masses of 90 and 100 kDa copurified with p53; from the human and monkey cell lines, only one protein of 90 kDa was copurified. All of the copurified proteins were identified as SV40 T antigen by Western blot analysis using anti-T-antigen antibody Pab 108 (Fig. 1B). From the SCID mouse cell line, the 100-kDa T antigen copurified in stoichiometric amounts with the 90-kDa form; from the SV-T2 mouse cell line, the 100-kDa protein was the major form of purified T antigen. Two lines of evidence suggest that the 100-kDa protein is a previously identified form of super T. First, the identification of this band as T antigen was further confirmed by sequence analysis of peptides resulting from the digestion of this protein (data not shown). Second, a form of super T with the same molecular mass as the 100-kDa T antigen that we observed has been identified in SV-T2 cells (40).

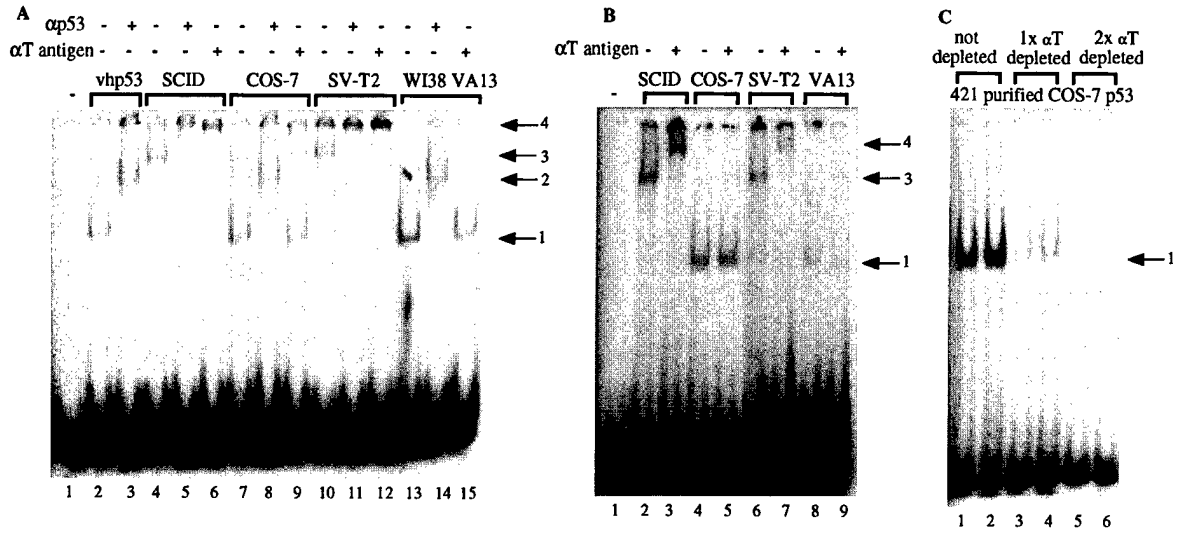


FIG. 2. p53 copurified with T antigen can bind to a DNA probe in EMSA. (A) A 200-pg aliquot of radiolabeled probe containing the p53-binding site from RGC was incubated with approximately 50 ng of vhp53 purified from HeLa cells or 50 ng of each of the protein samples shown in Fig. 1A and 100 ng of antibody as indicated for 30 min at 30°C prior to electrophoresis on a 3% polyacrylamide gel. Arrows indicate positions of retarded complexes: 1, DNA-p53; 2, DNA-p53-N19 (αp53); 3, DNA-p53-T antigen; 4, DNA-p53-T antigen-N19 or Pab 108 (αT antigen). (B) Like panel A but with a probe containing the p53-binding site identified in the p21 promoter. The gels shown in panels A and B were subjected to electrophoresis for 3 and 4 h, respectively, which accounts for the more advanced migration of the bands in panel B. (C) Like panel A but with increasing amounts (50 and 100 ng) or equivalent volumes of p53-T antigen complex purified from COS-7 cells with Pab 421 either before (lanes 1 and 2) or after one (lanes 3 and 4) or two (lanes 5 and 6) rounds of immunodepletion with anti-T-antigen antibody Pab 108.

As stated above, T antigen interacts with p53 and is thought to inhibit its binding to DNA. Therefore, when the p53 samples shown in Fig. 1A were used in EMSA with a probe containing the p53 *cis* element identified in the RGC, the results were unexpected (Fig. 2A). Vaccinia virus-expressed human p53 (vhp53) purified from HeLa cells was used as a T-antigen-minus control. It produced a retarded p53-DNA complex (lane 2) which was supershifted by the addition of anti-p53 antibody (lane 3). p53 purified from the monkey cell line COS-7 and the human cell line WI38 VA13 formed complexes similar in mobility to those formed by vhp53 (compare lane 2 to lanes 7 and 13). These complexes could be supershifted by the addition of anti-p53 antibody but not by the addition of anti-T-antigen antibody (cf. lanes 8, 9, 14, and 15). Western blot analysis was performed to ensure that equivalent amounts of p53 were present in all samples (data not shown). This result suggested that although p53 was purified in a complex with T antigen, it could bind DNA and that when bound to DNA, the p53 was no longer complexed with T antigen. p53 purified from the mouse cell lines SCID and SVT2, however, formed a complex with the DNA probe that migrated more slowly than the control p53-DNA complex (compare lane 2 to lanes 4 and 10). The addition of both anti-p53 antibody and anti-T-antigen antibody supershifted this complex (lanes 5, 6, 11, and 12), suggesting that the mouse p53-T antigen complex remained intact during the gel electrophoresis. This result indicated that the DNA-binding ability of mouse p53 was also unaffected by T antigen, but when purified from mouse cells, the p53-T antigen complex remained intact when bound to DNA.

To determine if these results were peculiar to the *cis* element identified in the RGC, further EMSA was performed with a probe containing the p53 element identified in the p21 promoter. Similar results were obtained (Fig. 2B), again demonstrating that p53-T antigen complexes purified from the mouse cell lines SCID and SV-T2 can bind DNA in the presence of T antigen (lanes 2, 3, 6, and 7) while those purified from the monkey and human cell lines bind DNA in the absence of T antigen (lanes 4, 5, 8, and 9). It was possible that excess p53 not bound to T antigen present in the p53-T antigen preparation from the human and monkey cell lines was responsible for the p53-DNA shift observed in EMSA, even though visualization by silver staining indicated that the ratio of p53 to T antigen was approximately 1:1 (Fig. 1A). To test this possibility, Pab 421-purified p53-T antigen complex from COS-7 cells was immunodepleted with anti-T-antigen antibody. Analysis by SDS-PAGE followed by silver staining indicated that immunodepletion resulted in the equal loss of both p53 and T antigen from the sample (data not shown). Importantly, EMSA revealed no DNA binding with the twice-immunodepleted sample (Fig. 2C; compare lanes 1 and 2 to lanes 5 and 6). This result strongly argues that the p53 DNA binding observed was not due to the presence of excess unbound p53. Taken together, these results suggest the existence of alternative mechanisms for the inhibition of p53-dependent transcription by T antigen. In addition, they suggest that the mechanism for inhibition of p53 may differ between mouse cells and human and monkey cells.

The p53-T antigen complex purified from mouse cells is transcriptionally inactive. We next wanted to determine whether the mouse p53-T antigen complex, which could bind to DNA, could also support p53-dependent transcription. The complex was immunopurified from SCID cells; as a control, SCID p53 was also purified in the presence of 2 M urea in order to dissociate T antigen. The resulting p53 samples are shown in Fig. 3A. When tested by EMSA, the p53-T antigen complex resulted in the slowly migrating band in which both p53 and T antigen were present (Fig. 3B, lanes 4 to 8). In contrast, the p53 protein purified in the presence of 2 M urea produced a retarded complex that was similar in mobility to that produced by the control vhp53 (compare lanes 9 to 12 to lanes 2 and 3) and did not react with the anti-T-antigen antibody Pab 108 (lane 13).

The ability of these p53 samples to stimulate transcription was next tested in an in vitro transcription assay as described previously (26). Unfractionated HeLa cell nuclear extract and

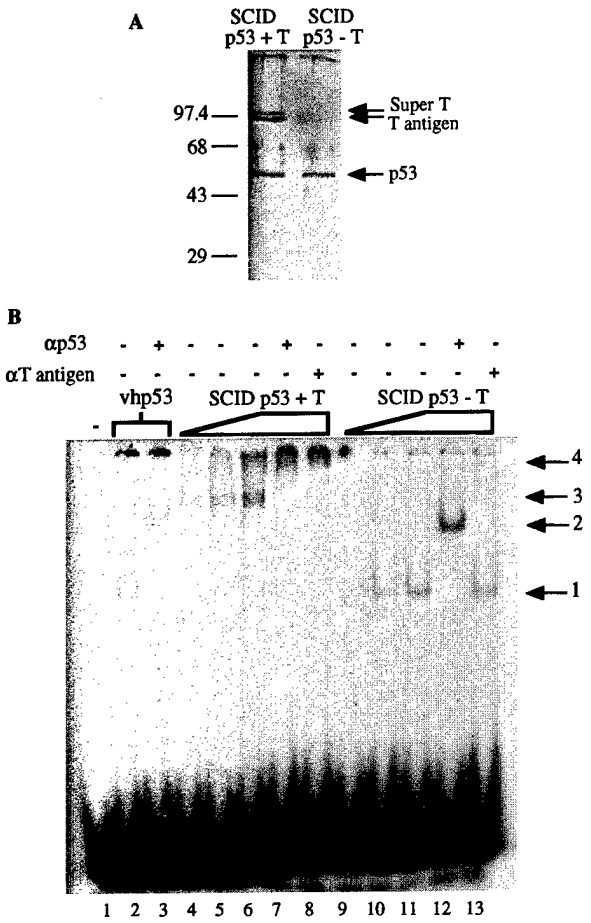


FIG. 3. Mouse p53 binds to DNA as a complex with T antigen in EMSA. (A) Silver-stained SDS-polyacrylamide gel of p53 purified from SCID cells with (+ T) or without (− T) T antigen. p53 was purified in the absence of T antigen by washing the p53-T antigen complex when bound to Pab 421 antibody linked to protein A-Sepharose beads with a 2 M urea solution. Sizes are indicated in kilodaltons. (B) Approximately 50 ng of vhp53 or increasing amounts (25, 50, and 75 ng) of the proteins used for panel A were incubated for 30 min at 30°C with 200 pg of radiolabeled probe containing the p53-binding site from RGC with 100 ng of antibody as indicated prior to electrophoresis on a 3% polyacrylamide gel. Arrows indicate positions of retarded complexes: 1, DNA-p53; 2, DNA-p53-N19 (αp53); 3, DNA-p53-T antigen; 4, DNA-p53-T antigen-N19 (αp53) or Pab 108 (αT antigen).

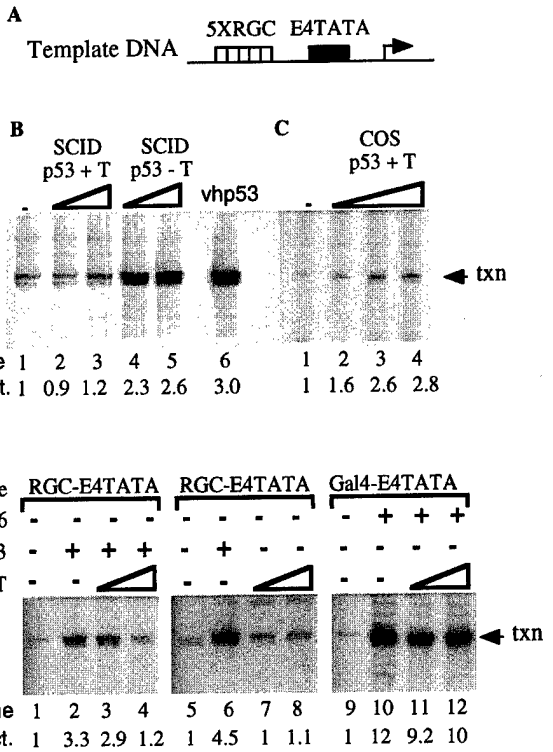


FIG. 4. Mouse p53-T antigen complex is transcriptionally inactive, while human p53-T antigen complex retains activity. (A) Schematic diagram of template DNA used in in vitro transcription reactions. (B) In vitro transcription reaction using HeLa nuclear extract (lane 1) with increasing amounts (300 and 600 ng) of SCID p53 purified with (lanes 2 and 3) or without (lanes 4 and 5) T antigen. vhp53 (200 ng) was used as a positive control (lane 6). Transcription (txn) products were subjected to electrophoresis on a 10% acrylamide-urea gel and visualized with a PhosphoImager using Adobe Photoshop software. (C) Like panel B but with increasing amounts (250, 500, and 750 ng) of COS-7 p53-T antigen complex (lanes 2 to 4). (D) Like panel B but with 100 ng of vhp53 (lanes 2 to 4 and 6) and increasing amounts (100 [lanes 3 and 7] and 400 [lanes 4 and 8] ng) of baculovirus-expressed T antigen (Bacl. T). In lanes 9 to 12, in vitro transcription was performed with a promoter similar to that used for panel A but with the RGC elements replaced with GAL4-binding sites. GAL4-VP16 (100 ng) was added to activate transcription (lanes 10 to 12) in the presence of increasing amounts (100 and 400 ng) of baculovirus-expressed T antigen (lanes 11 and 12).

a promoter template containing five p53 DNA-binding sites positioned immediately upstream of the adenovirus E4 TATA box (Fig. 4A) were incubated in the absence and presence of increasing amounts of p53. In the absence of exogenously added p53, the nuclear extract supported a low level of basal transcription (Fig. 4B, lane 1). The addition of vhp53 resulted in a threefold stimulation of transcription above this basal level (lane 6). By comparison to the control vhp53, addition of the mouse p53-T antigen complex isolated from SCID cells did not enhance transcription over basal levels (compare lane 6 to lanes 2 and 3) even though it bound DNA in EMSA. When the T-antigen proteins were removed, however, mouse p53 activated transcription 2.6-fold over basal levels (compare lane 1 to lanes 4 and 5). This indicates that loss of p53 activity was due to inhibition by T antigen and that the p53 was fully active on its own. These results were reproducible and therefore strongly suggest that in mouse cells, T antigen may inhibit p53-dependent transcription by blocking the transactivation domain of p53 and not by preventing it from binding to its cognate *cis* element.

Interestingly, COS-7 p53, which also purified in a complex with T antigen (Fig. 1) but bound DNA independently of T antigen (Fig. 2), retained the ability to activate p53-dependent transcription 2.8-fold over basal levels (Fig. 4C; compare lane 1 to lanes 2 to 4). This surprising result suggests that in monkey cells, T antigen may not necessarily inhibit p53 function despite its association with p53. T antigen can itself function as an activator of transcription from a simple promoter consisting of certain TATA elements and one upstream transcription factor-binding site (13, 33). Therefore, we performed assays in the presence of baculovirus-expressed purified T antigen to determine if T antigen alone could activate transcription in this system (Fig. 4D). p53-dependent transcription was inhibited when a fourfold excess of baculovirus-expressed T antigen was added to vhp53 (cf. lanes 2 and 4). The effect of T antigen on basal transcription was tested in assays using the amount of baculovirus T antigen sufficient to inhibit p53-dependent transcription. Transcription was not affected by the addition of T antigen alone (cf. lanes 7 and 8), suggesting that the transcription observed in Fig. 3C is p53 dependent and not T antigen

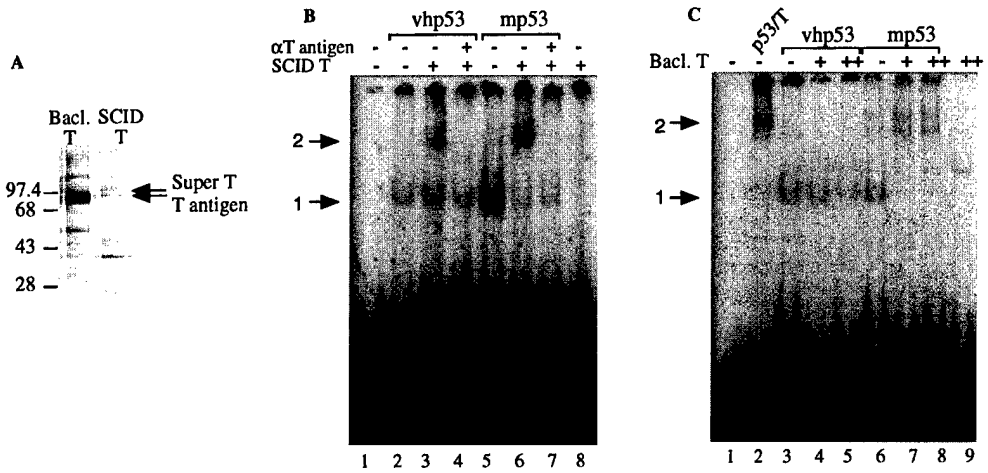


FIG. 5. Both mouse p53 and mouse T/super T contribute to the formation of a p53-T antigen complex that can bind to DNA. (A) Silver-stained SDS-polyacrylamide gel of baculovirus-expressed T antigen purified with anti-T-antigen antibody Pab 108 from SF21 insect cells (Bacl. T) and T antigen purified from stably transformed mouse SCID cells in the absence of p53 as described in Materials and Methods (SCID T). Sizes are indicated in kilodaltons. (B) Approximately 50 ng of vhp53 or 100 ng of SCID p53 (no T antigen present; mp53) was incubated for 30 min at 30°C with 200 pg of radiolabeled probe containing the p53 binding site from RGC, 50 ng of T antigen purified from SCID cells, and 100 ng of antibody (αT) as indicated prior to electrophoresis on a 3% polyacrylamide gel. DNA-protein complexes were visualized with a PhosphoImager using Adobe Photoshop software. Arrows indicate positions of retarded complexes: 1, DNA-p53; 2, DNA-p53-T antigen. (C) Like panel B but with 100 ng of SCID p53-T antigen complex (lane 2), 100 ng of vhp53, or 100 ng of SCID p53 (no T antigen present; mp53) incubated with 50 or 100 ng of baculovirus-expressed T antigen as indicated. Arrows indicate positions of retarded complexes: 1, DNA-p53; 2, DNA-p53-T antigen.

dependent. Finally, the effect of T antigen on activated transcription driven by bacterially expressed GAL4-VP16 was also tested in assays using template DNA containing five upstream GAL4-binding sites fused to the adenovirus TATA box. The addition of GAL4-VP16 to these reactions resulted in a 12-fold increase in transcription levels. This transactivation was minimally affected by the addition of baculovirus T antigen (compare lane 10 to lanes 11 and 12). Therefore, unbound T antigen does not affect transcription in these assays.

DNA binding by a p53-T antigen complex is species dependent. Next we wanted to determine what contributed to the different activities of the p53-T antigen complexes purified from either the mouse or the human and monkey cell lines. The difference may depend on the p53 present in these cell lines. At the amino acid level, monkey p53 is 96% identical to human p53, while mouse p53 is only 79% identical to human p53. Therefore, differences at the amino acid level or in protein modification may account for distinct interactions of mouse and human or monkey p53 with T antigen, with subsequent effects on p53-T antigen complex activity. Alternatively, the difference may be due to the presence of super T in the mouse cell lines but not in the human and monkey cell lines.

To address this issue, we performed EMSA with vhp53 and mouse p53 purified from SCID cells in the absence of T antigen. These samples were incubated with either a baculovirus-expressed T antigen (a 90-kDa protein) or T antigen purified from mouse SCID cells (90- and 100-kDa proteins [Fig. 5A]). Ideally, a preparation of the 100-kDa form of super T alone would have been used in these experiments, but to our knowledge no clone that only makes super T is available. Results are shown in Fig. 5B and C. The addition of T antigen/super T purified from SCID cells to mouse p53 resulted in the generation of a supershifted complex (Fig. 5B, lane 6). This complex was abolished by the addition of anti-T-antigen antibody (lane 7). T antigen/super T alone did not bind to DNA (lane 8). The addition of the same amount of T antigen/super T to human p53 also resulted in the generation of a supershifted complex (lane 3). However, we consistently observed that less human p53 than mouse p53 was supershifted under these conditions (cf. lanes 3 and 6), suggesting that T antigen/super T can form a DNA-binding complex more efficiently with mouse p53 than with human p53.

When baculovirus T antigen was used in these experiments, a supershifted complex was formed only with mouse p53 (Fig. 5C; compare lane 6 to lanes 7 and 8). Two slowly migrating complexes, possibly due to incomplete renaturation of the mouse p53 after the harsh conditions of the purification procedure, were observed. The slower-migrating complex migrated at a position similar to that of the complex formed by the mouse p53-T antigen complex immunopurified from the SCID cell line (lane 2), and addition of both anti-p53 antibody and anti-T-antigen antibody supershifted this slower-migrating complex (data not shown). In comparison to mouse p53, DNA binding of human p53 was inhibited by baculovirus T antigen (compare lane 3 to lanes 4 and 5 [1.4- and 1.9-fold reduction in binding, respectively]). Baculovirus T antigen alone did not bind specifically to DNA (lane 9). Therefore, in these in vitro assays, the DNA-binding ability of human p53 was inhibited by the addition of baculovirus-expressed T antigen, in accordance with previous observations (1). This result concurs with the data from in vitro transcription assays using vhp53, where transcription was inhibited by the addition of baculovirus-expressed T antigen (Fig. 4D, lanes 2 to 4). The fact that transcription and DNA binding are observed with p53-T antigen complex purified from COS-7 monkey cells suggests that there may be an important difference between this in vivo complex and that prepared in vitro by using virally expressed proteins. In addition, the molar ratio of T antigen to p53 may be higher in the in vitro complex, therefore affecting its activity.

The EMSA results presented in Fig. 5 suggest that mouse p53 is different from human p53 in its ability to interact with T antigen. Indeed, this difference was also noted when we attempted to purify p53-T antigen complex from monkey and mouse cells by using anti-T-antigen antibody. The complex remained intact when purified under stringent conditions from the SCID mouse cell line but dissociated when purified from the monkey COS-7 cell line (data not shown), suggesting that T antigen binds strongly to mouse p53 and weakly to human

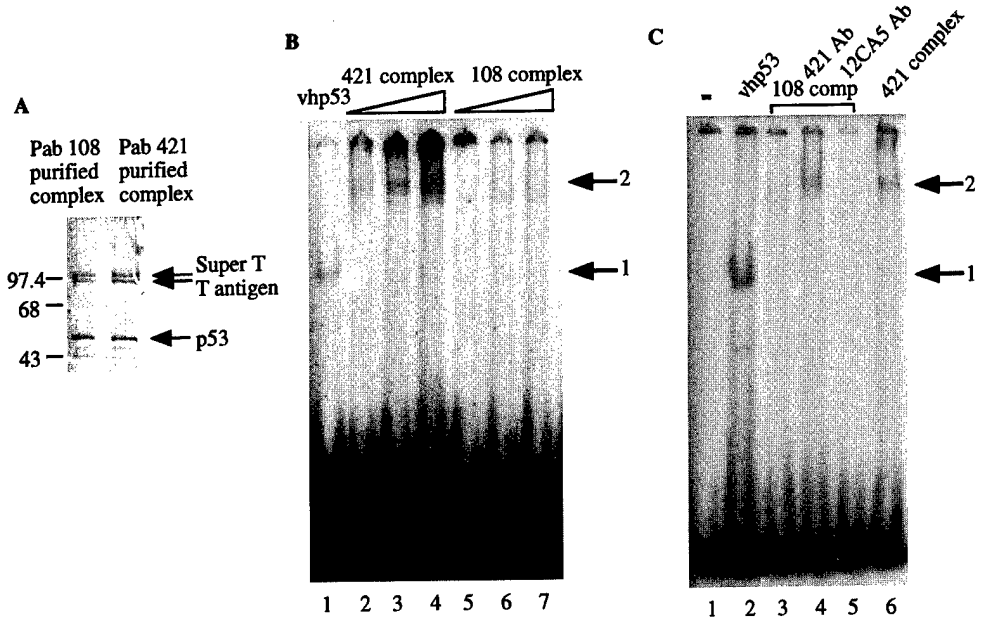


FIG. 6. Purification of p53-T antigen complex by Pab 421 is required for DNA-binding ability. (A) Silver-stained SDS-polyacrylamide gel of p53-T antigen complex purified from mouse SCID cells by using Pab 108 (anti-T antigen) or Pab 421 (anti-p53). Sizes are indicated in kilodaltons. (B) vhp53 (50 ng), increasing amounts (20, 40, and 60 ng) of Pab 421-purified SCID p53-T antigen complex, or increasing amounts (50, 75, and 100 ng) of Pab 108-purified complex were incubated for 30 min at 30°C with 200 pg of radiolabeled probe containing the p53-binding site from RGC prior to electrophoresis on a 3% polyacrylamide gel. Arrows indicate positions of retarded complexes: 1, DNA-p53; 2, DNA-p53-T antigen. (C) Like panel B but with 50 ng of vhp53, 100 ng of Pab 108-purified SCID p53-T antigen complex, or 50 ng of Pab 421-purified complex as indicated. In lanes 4 and 5, Pab 108-purified complex (108 comp) was preincubated for 20 min at 4°C with 5 μg of Pab 421 or 5 μg of antibody 12CA5 in 0.1 M D buffer, as indicated.

p53. Therefore, mouse p53, in addition to mouse T/super T, may contribute to the formation of a DNA-binding p53-T antigen complex.

An active conformation of p53 is required for p53-T antigen complex DNA binding. Wild-type p53 can be converted from an inactive or latent state to an active state that binds DNA (17). The latent state is thought to be dependent on a C-terminal negative regulatory domain within p53 that is proposed to interact with a motif in the core of the p53 tetramer, thereby forming a conformationally inactive complex (18). A number of conditions that modify the C terminus of p53 which convert p53 from a latent to an active state have been described. These include anti-p53 antibody Pab 421 (17, 18), phosphorylation (16, 17), acetylation (14), short single strands of DNA (19), and the redox/repair protein Ref-1 (20). The biological relevance of this model of activation is demonstrated by the fact that UV-induced activation of the transcriptional function of p53 does not require an increase in p53 protein levels (18).

The p53-T antigen complexes used in the previous experiments were purified by using Pab 421 antibody. This antibody recognizes an epitope in the C terminus of p53 and is thought to convert p53 from its latent to its active state, thereby significantly increasing its DNA-binding activity (18, 31). Consequently, we wanted to determine if this method of purification affected the DNA-binding ability of the complex. First, the mouse p53-T antigen complexes eluted from Pab 421 beads by using a 50% EG solution and 421 peptide were compared. In both cases, similar binding affinities were observed in EMSA (data not shown), indicating that binding was not dependent on the presence of 421 peptide. Next, we attempted to purify the complex by using p53-specific antibodies D0-1 and Pab 1801 (Santa Cruz), both of which recognize amino-terminus epitopes in human p53. However, in each case control experiments indicated that the stringent conditions required to elute p53 from these antibodies resulted in DNA-binding-deficient protein. Therefore, the complex was purified by using anti-T-antigen antibody Pab 108, which is not known to activate p53, and eluted with 50% EG solution, which does not affect the DNA-binding ability of p53 (36a). This method of purification resulted in a complex similar to that purified with Pab 421 when analyzed by SDS-PAGE followed by silver staining (Fig. 6A). However, the complex purified with Pab 108 had a significantly reduced affinity for DNA compared to that purified with Pab 421 (Fig. 6B; compare lanes 2 to 4 to lanes 5 to 7), suggesting that an active conformation of p53 may be required for p53-T antigen complex DNA-binding ability. To test if this was the case, we incubated the Pab 108-purified complex with Pab 421 prior to EMSA and then observed DNA binding (Fig. 6C; cf. lanes 3 and 4). The resulting shifted band migrated parallel with that produced by the Pab 421-purified complex (cf. lanes 4 and 6) and could be supershifted by the addition of anti-p53 and anti-T-antigen antibodies (data not shown). The activation of binding was not observed following incubation with an unrelated antibody (lane 5) or with bovine serum albumin (data not shown). These results strongly argue that the DNA-binding ability of the complex is dependent on the active conformation of p53.

DISCUSSION

In this paper we present biochemical evidence that p53 isolated in a complex with T antigen can bind to DNA, provided that p53 is in an active conformation. We also demonstrate that p53 exhibits species-specific interactions with T antigen. In vitro, T antigen apparently dissociates from human p53 in the presence of DNA, resulting in a transcriptionally active form of p53 bound to DNA. In contrast, mouse p53 binds DNA in a

complex with T antigen, resulting in transcriptionally inactive p53. Our results indicate that this difference may be attributable to a difference between mouse and human p53 and, in addition, possibly to the presence of super T in the mouse cell lines. On the basis of our results, we conclude that T antigen, under certain conditions, may repress p53-dependent transcription by a mechanism in which the transactivation domain of p53 is inhibited while DNA binding is unaffected. This mechanism is different from the previous proposed mechanism in which T antigen inhibits p53 by preventing it from binding DNA (1, 36). Therefore, similar to the inhibition of p53 by MDM2, it seems that T antigen may inhibit p53 by more than one mechanism.

Long et al. (27) have shown, using nuclear extracts in EMSA, that a small fraction of p53 from SV40-transformed monkey and rat cells specifically binds to DNA as a complex with T antigen. Our results, however, differ from theirs in that we observe significant DNA binding by p53-T antigen complex purified from mouse cells. The reason for this discrepancy may be that we used purified p53 activated by Pab 421 antibody as opposed to crude nuclear extracts. However, we did not observe DNA binding by p53-T antigen complex when using crude nuclear extracts in EMSA, either in the presence or in the absence of Pab 421 (data not shown). This may have been because the concentration of p53 in the crude extract was too low for Pab 421 activation to occur or for DNA binding to be observed.

The observation that the transactivation domain of mouse p53 may be inhibited by T antigen while DNA binding is unaffected has several implications. First, the surface of p53 required for DNA binding must be accessible in the mouse p53-T antigen complex. The region of p53 that is required for T-antigen binding has been mapped to residues 126 to 218, a region within the core domain required for DNA binding (34). However, resolution of the crystal structure of a human p53 core domain-DNA complex has localized the region of p53 directly contacted by DNA to three structural elements which include the H2 helix (amino acids [aa] 278 to 286), the L1 loop (aa 112 to 124), and the L3 loop (aa 236 to 251) (7). None of these elements overlap with the region required by p53 to interact with T antigen. Therefore, it is possible that p53 can concurrently form a complex with T antigen and bind DNA. Second, interaction of T antigen with the DNA-binding domain of p53 must either alter or block the activation domain of p53. Thus, it appears that the alteration of one domain on p53 may affect the function of the others.

Our results suggest that T antigen can form a transcriptionally inactive DNA-binding complex with mouse p53. The adenovirus E1B 55K protein also forms an inhibitory complex with p53 without displacing p53 from its cognate *cis* element (41). p53-mediated transcription is prevented because E1B 55K is a repressor of transcription which, via its interaction with p53, is targeted to p53-responsive genes. The mechanism of repression adopted by T antigen, however, is unlikely to be identical to that adopted by E1B 55K, as T antigen can function as a transcriptional activator (13, 33). T antigen is thought to activate transcription through direct interactions with both the basal transcription complex and upstream-bound transcription factors and may act like a component of TFIID by augmenting, or replacing, a function of $TAF_{II}250$ (9). Additionally, T antigen has been shown to enhance formation of TATA-binding protein–TFIIA complex on certain TATA elements (10). The fact that T antigen does not activate transcription when tethered to promoter DNA by p53 indicates that its activation function, as previously suggested (42), may be conformation dependent and that binding to p53 may alter its conformation.

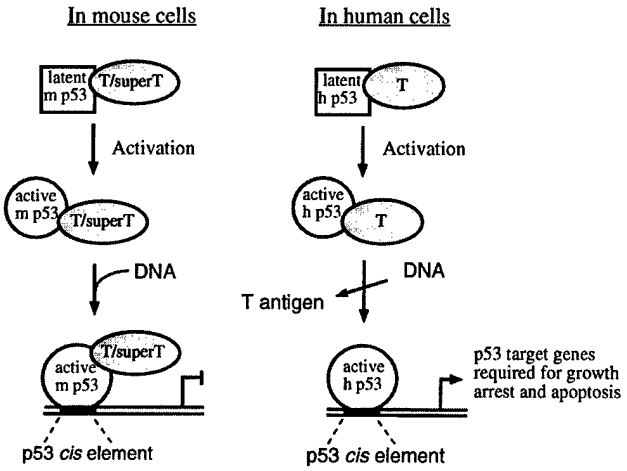


FIG. 7. Proposed model of p53 inhibition by T antigen in mouse (m) and human (h) cells.

Consistent with this view, GAL4-T antigen fusion protein has been shown to be transcriptionally inactive when brought to a target promoter bearing GAL4 DNA-binding sites (13). When bound as a complex with p53 to DNA, T antigen may inhibit p53-dependent transcription by steric hindrance of the activation domain of p53 or by deleteriously affecting the assembly or conformation of the basal transcription machinery. In addition, T antigen may interfere with the interaction of p53 with coactivators of transcription such as p300 (14).

Mouse cells are more readily transformed with SV40 than are human cells. Using the data presented in this paper, we propose a model which may explain the molecular mechanism of this difference (Fig. 7). In mouse cell lines, latent p53 and T antigen/super T form a complex that cannot bind DNA. Upon activation of p53, a conformational change in p53 may alter the p53-T antigen-super T complex such that DNA binding occurs. p53-responsive promoters would therefore be blocked by the transcriptionally inactive complex, and the tumor suppressor function of p53 would be reduced or lost. In human cell lines, latent p53 and T antigen also form a complex that is unable to bind to DNA. Again, upon activation of p53, a conformational change in p53 may alter the p53-T antigen complex such that DNA binding occurs. However, because human p53 appears to bind to DNA independently of T antigen and to retain the ability to activate transcription, p53 function would not be completely lost. Therefore, p53 target genes that are required for growth arrest and apoptosis would be activated in human cells containing T antigen and an active form of p53. This model relies on the fact that p53 must adopt an active conformation. Significantly, cellular factors that may activate p53 similarly to Pab 421 antibody, including protein kinases (16, 17), coactivator p300 (14), and the redox/repair protein Ref-1 (20), are found in the cells.

It is tempting to speculate that our model may also explain why SV40 is known to cause tumors in rodents (3, 8) but has not proven to do so in humans. Although recently this virus has been linked to some human cancers, a cause-and-effect relationship has not been established (4). Finally, it will be of interest to determine whether the differences reported here between species-specific forms of p53 are also applicable to interactions with other viral and cellular proteins. If so, such a distinction between rodent and primate p53 could have significant ramifications for the use of rodent models of transformation.

ACKNOWLEDGMENTS

We thank Arnold Berk and Carol Prives for providing cells and viruses, and we thank Francisco Renteria for help with cell culture. We also thank Frances Sladek, Noelle L'Etoile, Renee Yew, and members of the Liu laboratory for many helpful discussions and valuable comments on the manuscript.

This work was supported by grants CA75180-01 (X.L.) from the National Cancer Institute and DAMD17-96-1-6076 (X.L.) from U.S. Army Breast Cancer Research Program.

REFERENCES

1. **Bargonetti, J., I. Reynisdottir, P. Friedman, and C. Prives.** 1992. Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. Genes Dev. **6**:1886–1898.
2. **Butel, J. S., C. Wong, and B. K. Evans.** 1986. Fluctuation of simian virus 40 (SV40) super T-antigen expression in tumors induced by SV40-transformed mouse mammary epithelial cells. J. Virol. **60**:817–821.
3. **Carbone, M., P. Rizzo, and H. I. Pass.** 1997. Simian virus 40, poliovaccines and human tumors: a review of recent developments. Oncogene **15**:1877–1888.
4. **Carbone, M., P. Rizzo, P. M. Grimley, A. Procopio, D. J. Y. Mew, V. Shridhar, A. de Bartolomeis, V. Esposito, M. T. Giuliano, S. M. Steinberg, A. Levine, A. Giordano, and H. I. Pass.** 1997. Simian virus-40 large T-antigen binds p53 in human mesotheliomas. Nat. Med. **3**:908–912.
5. **Carey, M., J. Leatherwood, and M. Ptashne.** 1990. A potent Gal4 derivative activates transcription at a distance *in vitro*. Science **247**:710–712.
6. **Chen, S., M. Verderame, A. Lo, and R. Pollack.** 1981. Nonlytic simian virus 40-specific 100K phosphoprotein is associated with anchorage-independent growth in simian virus 40-transformed and revertant mouse cell lines. Mol. Cell. Biol. **1**:994–1006.
7. **Cho, Y., S. Gorina, P. D. Jeffrey, and N. P. Pavletich.** 1994. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science **265**:346–355.
8. **Cicala, C., F. Pompetti, and M. Carbone.** 1993. SV40 induces mesotheliomas in hamsters. Am. J. Pathol. **142**:1524–1533.
9. **Damania, B., and J. C. Alwine.** 1996. TAF-like function of SV40 large T antigen. Genes Dev. **10**:1369–1381.
10. **Damania, B., P. L. Lieberman, and J. C. Alwine.** 1998. Simian virus 40 large T antigen stabilizes the TATA-binding protein–TFIIA complex on the TATA element. Mol. Cell. Biol. **18**:3926–3935.
11. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcript initiation by RNA Pol II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
12. **El-Diery, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75**:817–825.
13. **Gruda, M. C., J. M. Zabolotny, J. H. Xiao, I. Davidson, and J. C. Alwine.** 1993. Transcriptional activation by simian virus 40 large T antigen: interactions with multiple components of the transcription complex. Mol. Cell. Biol. **13**:961–969.
14. **Gu, W., and R. G. Roeder.** 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell **90**:595–606.
15. **Haupt, Y., R. Maya, A. Kazaz, and M. Oren.** 1997. Mdm2 promotes the rapid degradation of p53. Nature **387**:296–299.
16. **Hupp, T. R., and D. P. Lane.** 1994. Regulation of the cryptic sequence-specific DNA-binding function of p53 by protein kinases. Cold Spring Harbor Symp. Quant. Biol. **59**:195–206.
17. **Hupp, T. R., D. W. Meek, C. A. Midgley, and D. P. Lane.** 1992. Regulation of the specific DNA binding function of p53. Cell **71**:875–886.
18. **Hupp, T. R., A. Sparks, and D. P. Lane.** 1995. Small peptides activate the latent sequence-specific DNA binding function of p53. Cell **83**:237–245.
19. **Jayaraman, L., and C. Prives.** 1995. Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell **81**:1021–1029.
20. **Jayaraman, L., K. G. K. Murthy, C. Zhu, T. Curran, S. Xanthoudakis, and C. Prives.** 1997. Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes Dev. **11**:558–570.
21. **Ko, L. J., and C. Prives.** 1996. p53: puzzle and paradigm. Genes Dev. **10**:1054–1072.
22. **Kubbutat, M. H. G., S. N. Jones, and K. H. Vousden.** 1997. Regulation of p53 stability by Mdm2. Nature **387**:299–303.
23. **Levine, A. J., J. Momand, and C. A. Finlay.** 1991. The p53 tumor suppressor gene. Nature **351**:453–456.
24. **Levine, A. J.** 1997. p53, the cellular gatekeeper for the growth and division. Cell **88**:323–331.
25. **Levitt, A., S. Chen, G. Blanck, D. George, and R. E. Pollack.** 1985. Two integrated partial repeats of simian virus 40 together code for a super-T antigen. Mol. Cell. Biol. **5**:742–750.
26. **Liu, X., and A. J. Berk.** 1995. Reversal of in vitro p53 squelching by both TFIIB and TFIID. Mol. Cell. Biol. **15**:6474–6478.
27. **Long, S.-B., H.-Y. Ho, C.-L. Chen, and M.-D. Lai.** 1995. Complex of simian virus large T antigen and p53 can bind DNA specifically. Anticancer Res. **15**:1375–1380.
28. **Lovett, M., C. E. Clayton, D. Murphy, P. W. J. Rigby, A. E. Smith, and F. Chaudry.** 1982. Structure and synthesis of a simian virus 40 super-T antigen. J. Virol. **44**:963–973.
29. **May, E., M. Kress, L. Daya-Grosjean, R. Monier, and P. May.** 1981. Mapping of the viral mRNA encoding a super-T antigen of 115,000 daltons expressed in simian virus 40-transformed rat cell lines. J. Virol. **37**:24–35.
30. **Miyashita, T., and J. C. Reed.** 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell **80**:293–299.
31. **Mundt, M., T. Hupp, M. Fritsche, C. Merkle, S. Hansen, D. Lane, and B. Groner.** 1997. Protein interactions at the carboxyl terminus of p53 result in the induction of its in vitro transactivation potential. Oncogene **15**:237–244.
32. **Oliner, J. D., J. A. Pietenpol, S. Thiagalingam, J. Gyuris, K. W. Kinzler, and B. Vogelstein.** 1993. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature **362**:857–860.
33. **Rice, P. W., and C. N. Cole.** 1993. Efficient transcriptional activation of many simple modular promoters by simian virus 40 large T antigen. J. Virol. **67**:6689–6697.
34. **Ruppert, J. M., and B. Stillman.** 1993. Analysis of a protein-binding domain of p53. Mol. Cell. Biol. **13**:3811–3820.
35. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63**:1129–1136.
36. **Segawa, K., A. Minowa, K. Sugasawa, T. Takano, and F. Hanaoka.** 1992. Abrogation of p53-mediated transactivation by SV40 large T antigen. Oncogene **8**:543–548.

36a. **Sheppard, H. M., and X. Liu.** Unpublished results.

37. **Smith, A. F., R. Smith, and E. Pouche.** 1979. Characterization of different tumor antigens present in cells transformed by simian virus 40. Cell **18**:335–346.
38. **Storey, A., M. Thomas, A. Kalita, C. Harwood, D. Gardiol, F. Mantovani, J. Breuer, I. M. Leigh, G. Matlashewski, and L. Banks.** 1998. Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. Nature **393**:229–234.
39. **Thut, C. J., J. A. Goodrich, and R. Tjian.** 1997. Repression of p53-mediated transcription by MDM2: a dual mechanism. Genes Dev. **11**:1974–1986.
40. **Wilson, V. G., and R. L. Williams.** 1985. Origin binding by a 100,000-dalton super-T antigen from SVT2 cells. J. Virol. **56**:102–109.
41. **Yew, P. R., X. Liu, and A. J. Berk.** 1994. Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. Genes Dev. **8**:190–202.
42. **Zhu, J., M. Abate, P. W. Rice, and C. Cole.** 1991. The ability of simian virus 40 large T antigen to immortalize primary mouse embryo fibroblasts cosegregates with its ability to bind p53. J. Virol. **65**:6872–6880.